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(71) Applicant: MAXIM PHARMACEUTICALS, INC. [US/US]; Suite 400, 8899 University City Lane, San Diego, CA 92122 (US).

(72) Inventors: EWALT, Karla, L.; 13296 Kingsfield Court, San Diego, CA 92130 (US). HANDLEY, Harold, H., Jr.; 705 Arden Drive, Encinitas, CA 92024 (US).

(74) Agent: ALTMAN, Daniel, E.; Knobbe, Martens, Olson and Bear, LLP, 16th floor, 620 Newport Center Drive, Newport Beach, CA 92660 (US).

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(54) Title: RECOMBINANT CtB–BASED VACCINES

(57) Abstract

The present invention relates to a method of preparing conjugates between a carrier component and an immunogenic component in a time and temperature dependent manner. The conjugated material delivers the immunogenic component or components to the mucosal immune system. The present invention also relates to a composition of matter comprising carrier and immunogenic components. More specifically, the present invention relates to a composition of matter containing the specific mucosal delivery properties of CtB and diphteria toxoid.

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RECOMBINANT CtB-BASED VACCINES

BACKGROUND OF THE INVENTION

The Director-General of the World Health Organization recently stated that vaccination is the most cost effective health intervention available and that prevention of disease is better than a cure. Although there are a significant number of vaccines currently available to protect humans and other subjects from infectious disease, the variety of serious pathogens far outnumbers the diversity of effective vaccines currently available. The majority of pathogens contact and enter humans through the mucosal tissues, those of the mouth, eyes, respiratory, intestinal and urogenital tracts. Yet few vaccines have been successful at eliciting immune protection within these mucosal tissues, despite the fact that a majority of immune cells reside in these tissues.

Vaccine creation often involves the sequential identification, isolation, and formulation of a pathogen of interest, which is then used to vaccinate a host. The vaccination, if successful, generates a protective response in the immunized host organism. A key step in creating an effective vaccine is to present enough of the pathogen to the host so that the host's immune system will mount a protective immune response without permitting the pathogen to harm the immunized host organism.

There are a number of methods and vaccine types known for accomplishing this task. For example, "live" vaccines are compositions that contain a pathogen of interest that is alive, but attenuated so that it is incapable of causing serious disease in a host during the immunization procedure. In contrast, "killed" vaccines are compositions that isolate a pathogen of interest and then subject the isolated material to conditions that kill or inactivate the pathogens and still induce protective immunity.

One method of generating a killed vaccine is to expose the pathogen of interest to a fixing reagent. Formaldehyde is a frequently used agent. For example, influenza and polio vaccines have been generated where virus particles are treated with formaldehyde, before administration. Vaccines against other pathogens have been generated using formaldehyde, either alone or in combination with other detoxifying agents. For example, U.S. Patent No. 4,017,360 discusses a hepatitis B antigen (HBsAg) useful as a vaccine component, where isolated HBsAg is treated with pepsin or optionally treated with formaldehyde at a concentration of 50 to 200 micrograms per ml of solution to inactivate any viruses remaining in the solution. The solution is incubated for 50 to 100 hours at a temperature from about 30 to 38°C. U.S. Patent No. 4,291,020 discusses a method of generating a non-A, non-B hepatitis vaccine by treating the disease causing agent with formalin (4% formaldehyde solution) at a dilution of 1:1,000-1:10,000. The duration of treatment is from 24-120 hours with preferred conditions being 96 hours (4 days) at 37°±4°C. The formalin-treated inactivated agent, or portions of the agent, can be later used to produce a vaccine against non-A, non-B hepatitis.

In U.S. Patent Nos. 4,374,127 and 4,452,734, a method for preparing herpes virus subunit antigens suitable for vaccine use is discussed. This patent suggests the use of formalin, a formaldehyde solution typically around 4%,

and other sterilizing compounds, to detoxify a preparation for use in a vaccine. The method described discusses the optional use of formalin at a concentration of 100 μg/ml with an incubation step of 72 hours at 36°C.

U.S. Patent No. 4,997,915 discusses a vaccine against *Bordatella pertussis* (*B. pertussis*) infection using glutaraldehyde or formaldehyde cross-linking reagents to detoxify the *B. pertussis* toxins lymphocytosis promoting factor (LPF) and filamentous hemaglutinin (FHA). The resulting purified and detoxified LPF and FHA can be used to formulate a vaccine against the pertussis toxins. For example, in one embodiment of U.S. Patent No. 4,997,915, 500 μg/ml of protein was heated to 37°C and detoxified with formaldehyde at a final concentration of 0.25% v/v. The solution was incubated at 37°C for 6 weeks. Also, U.S. Patent No. 5,578,308 disclosed a detoxified *B. pertussis* toxin preparation created through a two step process where the toxin was first partially detoxified using gluteraldehyde, and then finally detoxified using formaldehyde added to the antigen and an amino acid solution at 1 to 10% by weight. The formalin was added over a 3 to 10 day period at a temperature between 37 and 43°C.

In U.S. Patent No. 5,453,273, a ringworm vaccine comprising an effective amount of a homogenized, formaldehyde-killed *Microsporum canis* culture in a carrier was discussed. A 0.2% formaldehyde solution was used to kill *M. canis* using a four day incubation period at room temperature.

Many pathogens cause disease by first colonizing or penetrating through the mucosa of the gastrointestinal, respiratory, or genital tract. Elevating the levels of local IgA and IgG antibodies at these mucosal surfaces is believed to be central to the primary defense against these pathogens. "Neutralizing" antibodies against these pathogens are known to prevent adherence and colonization of bacteria at the mucosal surfaces and have been shown to detoxify microbial toxins. Systemic vaccination fails to stimulate IgA production and infrequently increases IgG antibodies in the mucosa of the intestinal tract or other mucosal sites. In contrast, mucosal immunization can be shown to induce high titer IgA and IgG at the local site of vaccination. IgA is considered the hallmark of the mucosal immune response. IgA constitutes approximately 90% or more of the immunoglobulin from jejunal fluid (gut) or breast milk as opposed to only 10% of serum immunoglobulin. (Handbook of Mucosal Immunology, eds. Ogra, et al., (1994)).

In addition to creating a vaccine preparation, a protocol for immunization is required to maximize the effectiveness of the preparation. A major element of consideration in such a protocol is the route of administration. Two of the most attractive routes of mucosal vaccination in humans are the oral and nasal routes.

Oral vaccines have recently been developed and licensed for human use. One example of such an oral vaccine is an oral cholera vaccine containing cholera toxin B-subunit (CtB) together with a killed whole-cell vaccine component. (Holmgren & Svennerholm, *Gast. Clin. N. Am.*, 21:283-302 (1992)). CtB is a well characterized non-toxic, yet potent mucosal immunogen, partly relating to its high-affinity binding to the receptor GM1 ganglioside. (Holmgren, J., *Nature*, 292:413-417 (1981)). In order to induce a strong local immune response after vaccination, a whole cell preparation or a protein with mucosa-binding properties are required. (Aizpurua & Russell-Jones, *J. Exp. Med.*, 167:440-451 (1988)). Consistent with this notion, several studies in animals have shown that CtB used as a carrier for various protein or carbohydrate antigens can markedly enhance the mucosal immunogenicity for antigens that are otherwise not immunogenic. (Holmgren, et al., *Am. J. Trop. Med. Hyg.*, 50(5)(Suppl.):42-54 (1994); Bergquist, et al., *Infect. Immun.*,

63:2021-2025 (1995)). Also, U.S. Patent No. 5,182,109, describes the intranasal administration of a number of vaccine preparations mixed with CtB as an adjuvant to promote an immune respone. CtB has also been used in a conjugate vaccine administered intranasally. (Russell *et al., Infect. Immun.,* 64:1272-1283(1996)).

Earlier experiments in mucosal vaccination indicated that oral vaccination activated mucosal lymphocytes which subsequently localized to disparate mucosal tissues such as the mammary, salivary, respiratory and genital tissues in addition to the intestines. (Mestecky, J., *J. Clin. Immunol.,* 7:265-276 (1987)). More recent investigations however, suggest that this redistribution of immune cells results in a predominance of cells homing to the site of vaccination. (Quiding-Jäbrink, M., et al., *Infect. Immun.,* 63:853-857 (1995); Hopkins, S., et al., *Infect. Immun.,* 63:3279-3286 (1995)). The site of infection may then dictate the preferred route of vaccination. Thus, while the oral route may remain a more convenient and preferred route of mucosal vaccination, more effective routes of vaccination against respiratory or genital pathogens can be nasal or genital administration.

Recent studies have also shown that vaginal and cervical fluids possess a higher titer of specific antibodies when induced by urogenital vaccination than by oral vaccination. (Johansson, E.-L., et al., *Infect. Immun.,* 66:514-520 (1998); Di Tommaso, A., et al., *Infect. Immun.,* 64:974-979 (1996); Wassén, L., et al., *Scand. J. Immunol.,* 44:408-414 (1996)). Additionally, nasal immunization in animals has been superior to the oral route for stimulating local antibody production in the airway mucosa. (Hopkins, S., et al., *Infect. Immun.,* 63:3279-3286 (1995); Bergquist, et al., *Infect. Immun.,* 63:2021-2025 (1995)). Unexpectedly, nasal immunization has also been effective in priming the immune response in the genital mucosa of both animals and humans, and may prove an attractive route of vaccination against sexually transmitted diseases. (Bergquist, et al., *Infect. Immun.,* 65:2676-2684 (1997); Hopkins, S., et al., *Infect. Immun.,* 63:3279-3286 (1995); Johansson, E.-L., et al., *Infect. Immun.,* 66:514-520 (1998); Di Tommaso, A., et al., *Infect. Immun.,* 64:974-979 (1996); Russell, M.W., *Infect. Immun.,* 64:1272-1283 (1996)). This localized vaccination approach may prove most effective for inducing mucosal immunity through primary vaccination and/or subsequent vaccination at the primary site of anticipated infectious exposure.

A number of human studies have tested the efficacy of nasal vaccination. (Bergquist, et al., *Infect. Immun.,* 65:2676-2684 (1997); Moldoveanu, Z., et al., *Vaccine,* 13:1006-1012 (1995); Aggerbeck, H., et al., *Vaccine,* 15:307-316 (1997)). It is anticipated that the first approved nasal vaccine will be an influenza vaccine from Aviron, Inc. (Mountain View, CA). This vaccine may be the first in a growing trend, opening the door to a new approach to vaccination and a greater understanding of the relationships between vaccine formulation, mucosal immunization, and the preferred route of delivery.

Novel vaccine formulations and compositions that enhance the mucosal immune response would be a boon to medical science by providing new vaccine formulations specifically directed against pathogens which might evade systemic immunity through colonization of the non-serous fluids of the mucosa.

SUMMARY OF THE INVENTION

The present invention relates to a method of preparing conjugates between a carrier component and an immunogenic component in a time and temperature dependent manner. The conjugated material delivers the

immunogenic component or components to the mucosal immune system. The present invention also relates to a composition of matter comprising carrier and immunogenic components. More specifically, the present invention relates to a composition of matter containing the specific mucosal delivery properties of CtB and diphtheria toxoid.

One embodiment of the present invention is a vaccine preparation comprising an effective amount of a carrier component and an immunogenic component in a pharmaceutically acceptable carrier, wherein the carrier component and the immunogenic component are cross-linked for at least 2 weeks at a temperature less than 15°C, whereby the carrier component and an immunogenic component are cross-linked in a time and temperature dependent manner. In one aspect of this embodiment, the carrier component and the immunogenic component are cross-linked using an aldehyde selected from the group consisting of glutaraldehyde, formaldehyde, glyceraldehyde, acetaldehyde, phenylaldehyde, valeraldehyde, or 3,4-dihydroxyphenylacetaldehyde. In another aspect, the carrier component and the immunogenic component are cross-linked using a ketone selected from the group consisting of acetone, methyl ethyl ketone, or 3-pentanone.

A number of sources for the immunogenic components used in the vaccine compositions of the present invention. In one embodiment, the immunogenic component is selected from the group consisting of viruses, bacteria, fungi, proteins, polypeptides, glycoproteins, lipids, glycolipids, or immunogenic portions thereof. In another embodiment, the immunogenic component is selected from the group consisting of *Bordetella pertussis* toxin subunit S2, S3, S4, S5, Diphtheria toxin fragment B, *E. coli* fimbria K88, K99, 987P, F41, CFA/I, CFA/II (CS1, CS2, CS3), CFA/IV (CS4, CS5, CS6), and P fimbraiae.

A number of carrier components are contemplated for use in the present invention. In one embodiment, the carrier component is selected from the group consisting of cholera toxin, staphylococcal α-hemolysin toxin, the staphylococcal δ-hemolysin toxin, the *Vibrio cholerae* thermostable direct hemolysin toxin, the pertussis toxin, and the *E. coli* heat-labile enterotoxin. In another embodiment, the carrier component is a mucosa binding toxin subunit selected from the group consisting of the cholera toxin subunit A, the cholera toxin subunit B, and the *E. coli* heat-labile enterotoxin subunit B, or a portion mucosal membrane binding portion thereof.

A particular embodiment of the present invention is a vaccine preparation comprising an effective dose of diptheria toxin and cholera toxin subunit B in a pharmaceutically acceptable carrier, wherein the diptheria toxin and the cholera toxin subunit B are formaldehyde cross-linked for at least two weeks at an incubation temperature of no more than 20°C.

The present invention further contemplates a method of making a time and temperature cross-linked vaccine preparation comprising the steps of providing an immunogenic component and a carrier component, and cross-linking the immunogenic component and the carrier component for no less than two weeks at a temperature of no more than 4°C.

A number of cross-linking compounds are contemplated for use in the methods of the present invention. In one embodiment, the immunogenic component and the carrier component are cross-linked using an aldehyde selected

from the group consisting of glutaraldehyde, formaldehyde, glyceraldehyde, acetaldehyde, phenylaldehyde, valeraldehyde, or 3,4-dihydroxyphenylacetaldehyde. In another embodiment, the immunogenic component and the carrier component are cross-linked using a ketone selected from the group consisting of acetone, methyl ethyl ketone, or 3-pentanone.

The present invention contemplates using a number of pathogens and toxins for use as the immunogenic component of the claimed method. In one embodiment of the method, the immunogenic component is selected from the group consisting of *Bordetella pertussis* toxin subunit S2, S3, S4, S5, Diphtheria toxin fragment B, *E. coli* fimbria K88, K99, 987P, F41, CFA/I, CFA/II (CS1, CS2, CS3), CFA/IV (CS4, CS5, CS6), and P fimbraiae.

Similarly, a number of carrier components are contemplated for use in the methods of the present invention. An embodiment of the invention is where the carrier component is selected from the group consisting of cholera toxin, staphylococcal α-hemolysin toxin, the staphylococcal δ-hemolysin toxin, the *Vibrio cholerae* thermostable direct hemolysin toxin, the pertussis toxin, or the *E. coli* heat-labile enterotoxin. In another embodiment, the carrier component is a toxin subunit selected from the group consisting of the cholera toxin subunit A, the cholera toxin subunit B, and the *E. coli* heat-labile enterotoxin subunit B.

Additional reagents are contemplated for use in the method. One embodiment of the method further comprising a stabilizer. In one aspect of this embodiment, the stabilizer is added prior to cross-linking the immunogenic component and the carrier component. In another aspect, the stabilizer is added after cross-linking the immunogenic component and the carrier component.

Another embodiment of the present invention describes a method of making a vaccine preparation comprising the steps of providing diptheria toxin and cholera toxin subunit B, and conjugating the diptheria toxin and the cholera toxin subunit B with a formaldehyde solution for at least two weeks at 4°C, whereby a cross-linked diptheria-cholera toxin subunit B vaccine preparation is formed.

The present invention further contemplates a method of generating an immune response in a mammal with a time and temperature cross-linked vaccine preparation comprising the steps of administering the time and temperature dependent vaccine of Claim 1 to the mucosal tissues of the mammal in an amount sufficient to generate an immune response to the vaccine in the mammal. Embodiments of the present invention include modes of administration such as nasal, oral, rectal, vaginal, by inhalation, or by ophthalmic administration.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 shows a gel filtration protein elution (A_{280}) profile with coincident antigenic (anti-Dt) profile as determined by Gm-1 ELISA for CtB-Dt conjugate as discussed in Example 1.

FIGURE 2 shows the number of antigen (diphtheria toxin) specific antibody secreting cells infiltrating into the lungs and spleen of vaccinated animals as discussed in Example 1.

FIGURE 3 the diphtheria toxin specific antibody titers from tissue fluids isolated from the lung and small intestines of vaccinated animals as discussed in Example 1. Solid bars represent the immune response to Dt, open bars

represent the immune response to rCtB. The data are expressed as the geometric mean titer ± standard error of the mean.

FIGURE 4 shows the average diphtheria toxin specific IgG titer from the pooled sera of all animals from each group. The animals were bled prior to vaccination and thereafter at biweekly intervals as discussed in Example 1. Asterisks indicate vaccination times.

FIGURE 5 compares the antigenic titer of a freshly mixed CtB-Dt vaccine against a time and temperature conjugated CtB-Dt vaccine as discussed in Example 2. Open bars show the amount of CtB in each sample. Solid bars show the amount of CtB-Dt conjugate.

FIGURE 6 compares the antigenic titer of vaccines from Experiment 1 after incubation for 64 days at 4°C as discussed in Example 2. Open bars show the amount of CtB in each sample. Solid bars show the amount of CtB-Dt conjugate.

FIGURE 7 compares the effects of varied component ratio and time of incubation to the antigenic profile as discussed in Example 3. Solid circles (•) show Dt conjugate formation after 2 weeks; crosses (+) show conjugate formation after 4 weeks; solid triangles (▼) show Tt conjugate formation after 2 weeks; open squares (□) show Tt conjugate formation after 4 weeks.

FIGURE 8 shows the time dependence of cross-linking for two commercial diphtheria toxoid preparations to rCtB as discussed in Example 4. Open circles (○) show the amount of conjugates formed between the SBL Vaccin Dt and CtB. Solid circles (•) show the amount of conjugates formed between SSI Dt and CtB.

FIGURE 9 compares the effect of a range of incubation temperatures on rCtB-Dt vaccine antigenicity as discussed in Example 5. Open bars show the amount of CtB in each sample. Solid bars show the amount of CtB-Dt conjugate.

FIGURE 10 shows the effect of pH on the conjugation of Dt to rCtB as discussed in Example 6. The data are expressed as the average antigenic response measured as relative ELISA Units (EU) ± standard deviation.

FIGURE 11 compares the effects of various conjugation conditions on the yield of IgG and rCtB conjugates as discussed in Example 7. The data are expressed as the average antigenic response measured as relative ELISA Units (EU) ± standard deviation.

FIGURE 12 compares the effects of dilution, reduction by sodium borohydride, and the addition of ethanolamine on Dt and rCtB conjugates as discussed in Example 9. The data are expressed as the average antigenic response measured as relative ELISA Units (EU) ± standard deviation.

FIGURE 13 shows the effect of purification on the immunogenicity of the purified conjugated vaccine composition discussed in Example 11.

FIGURE 14 shows the extent of immune recognition of intranasal Dt as discussed in Example 12. Solid bars show the immune response directed against Dt, open bars show the immune response directed against rCtB. The data are expressed as the geometric mean titer ± standard deviation.

FIGURE 15 shows the effect of the vaccine composition discussed in Example 12 on lung tissue anti-Dt IgA and IgG. Open bars show the IgA titer and solid bars show the IgG titer for each vaccine composition tested. The data are expressed as the geometric mean titer ± standard deviation.

FIGURE 16 shows the amount of rCtB in µg/ml conjugated to *Bordetella pertussis* cells. FIGURE 16A shows the amount of carrier component detected from the time and temperature conjugated sample in the solid bars and the amount of *B. pertussis* antigen detected with open bars. FIGURE 16B compares various conjugation methods at the same component ratios on the amount of rCtB detected by the assay described in Example 13. The solid bar shows the amount of rCtB detected in the time and temperature conjugated sample, the shaded bar shows the amounts amount of rCtB detected in a vaccine composition conjugated using SPDP, and the open bar represents the amount of rCtB detected in a vaccine composition that was mixed (admixture). The data are expressed as the average of 3 measurements ± standard deviation.

FIGURE 17 shows the IgA (open bars) and IgG (solid bars) titers detected in lung tissue samples from animals vaccinated intranasally with vaccine compositions made using the method of the present invention and others as discussed in Example 13. The data are expressed as the geometric mean titer ± standard deviation.

FIGURE 18 shows the numbers of guinea pigs surviving after lethal diphtheria toxin challenge. Animals immunized three times with a time and temperature conjugated vaccine composition are represented by a (○). Animals immunized once with a time and temperature conjugated vaccine composition are represented by a (△). Animals immunized with a commercial vaccine composition are represented by a (●). Animals immunized with PBS are represented by a (◆)

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention relates to the production and use of vaccine compositions that generate long lasting, protective mucosal and systemic immunity to a variety of pathogens and their associated toxins. More specifically, the present invention relates to the use of wild-type (CtB) or recombinant cholera toxin subunit B (rCtB) as a carrier component conjugated to an immunogenic component using a time and temperature dependent method of cross-linking or conjugation. Vaccines manufactured with this method optimize the presentation of the immunogenic component to a host's immune system and elicit mucosal and systemic production of an immune response in vaccinated subjects beyond that expected with CtB conjugated vaccines found in the prior art.

The present invention contemplates a method of cross-linking a carrier component to a selected immunogenic component to generate vaccine compositions that stimulate mucosal as well as systemic immune responses. Vaccine compositions that stimulate only a mucosal immune response are also contemplated. Components for the vaccine compositions of the present invention comprise a carrier component that localizes the vaccine composition to the mucosa and an immunogenic component, such as a selected epitope or immunogen. These components are modified by the time and temperature conjugation method of the present invention to produce the disclosed vaccine compositions.

Generally, the method of generating the vaccine compositions of the present invention encompasses modifying, combining, and incubating the selected vaccine components at an optimal temperature for an optimal period of time and at an optimal pH, to create cross-links between the vaccine components.

A variety of methods for combining, conjugating, and cross-linking vaccine components are disclosed. The carrier component and the immunogeninc component can be first combined and then modified to induce cross-linking or conjugation. Alternatively, the vaccine components can be separately modified to induce cross-linking upon combination.

The vaccine components of the present invention are combined in a desired weight ratio so as to produce a vaccine that can elicit an optimal protective mucosal and systemic immune response from an immunized subject. In one embodiment of the present invention the carrier component and the immunogen component are mixed in a ratio in which the proportion of carrier component exceeds that of the immunogen, for example, at ratio of about 500,000:1. In another embodiment of the present invention, the carrier component and the immunogenic component are mixed in a ratio of about 100,000:1. In still another embodiment the adjuvant and the immunogen are mixed in a ratio of about 1000:1. And in yet another embodiment, the ratio can be that of 1:1. Alternatively, the components of the vaccine can also be combined in a ratio where the proportion of carrier component is less than that of the immunogen. For example, peptides conjugated to rCtB can be conjugated at a ratio of 5:1 or 10:1.

The vaccine components can be modified using a variety of methods to create the desired vaccine compositions in a time and temperature dependent manner. Any modification method that results in the cross-linking or conjugation of the vaccine components in a time and temperature dependent manner is contemplated by the present invention. The method of modification should yield conjugated carrier and immunogenic components that results in a vaccine composition with an increased immunogenicity or antigenicity as compared to vaccine compositions conjugated or produced using other methods.

In one aspect of the present invention, one or more of the vaccine components of interest can be modified to induce cross-linking or conjugation by treatment with an aldehyde. For example, suitable aldehydes include glutaraldehyde, formaldehyde, glyceraldehyde, acetaldehyde, phenylaldehyde, valeraldehyde, or 3,4-dihydroxyphenylacetaldehyde.

In another aspect of the present invention, ketones can be used to modify and cross-link the vaccine components. Suitable ketones would include acetone, methyl ethyl ketone, 3-pentanone, or any other ketone known to one of ordinary skill in the art.

Use of other cross-linking or conjugation agents is also contemplated, including *N*-succinimidyl 3-[2-pyridyldithio] propionate (SPDP), ultraviolet cross-linking and other protein cross-linking methods known in the art.

In one embodiment of the present invention, formaldehyde is used to modify the components of the vaccine to induce cross-linking or conjugation in a time and temperature dependent manner. In this embodiment, a purified or

partially purified carrier or immunogenic component, or both, can be modified with formaldehyde at an optimal concentration. In one embodiment the concentration of formaldehyde used to induce cross-linking is from about 0.01% to 15% (v/v). In another embodiment the concentration of formaldehyde used to induce cross-linking is from about 0.1% to 1% (v/v). In yet another embodiment the concentration of formaldehyde used to modify the components of the vaccine is about 0.1%.

The present invention further contemplates the addition of stabilizing agents to the vaccine component mix. These stabilizing agents act to stabilize the vaccine product after time and temperature dependent conjugation or to be useful in controlling the extent of cross-link formation between the vaccine components. For example, the addition of exogenous amino acids or amino containing compounds can serve as stabilizing agents.

The present invention contemplates performing the time and temperature conjugation reaction over a period of time from about 1 to 120 days. In another embodiment, the present invention contemplates performing the time and temperature conjugation reaction over a period of time from about 14 to 90 days. In still another embodiment, the present invention contemplates performing the time and temperature conjugation reaction over a period of time from about 28 to 60 days. In still a further embodiment, the time and temperature conjugation method of the present invention is performed for 30 days.

The present invention contemplates performing the time and temperature conjugation reaction over a range of temperatures. The temperatures used will depend on the stability of the immunogenic component chosen for conjugation with the carrier component. In one embodiment, the range of temperatures is from about -1°C to 37°C. In another embodiment, the present invention contemplates performing the time and temperature conjugation reaction over a range of temperatures from about 2-15°C. In still a further embodiment, the time and temperature conjugation method of the present invention could occur at 4°C.

The pH levels of the time and temperature conjugation reaction have also been shown to be important when generating the vaccine compositions of the present invention. Accordingly, the pH levels for performing the time and temperature conjugation method of the present invention should range from about pH 6.5 to pH 12. In another embodiment, the present invention contemplates performing the time and temperature conjugation reaction over a pH range from about 7 to 10. In still another embodiment, the present invention contemplates performing the time and temperature conjugation reaction over a pH range from about 7.2 to 8.5. In still another embodiment the time and temperature conjugation reaction is performed at a pH of 8.0.

Following incubation and formation of cross-links between the components of the vaccine, the product can be isolated, purified, and used to immunize a subject requiring the induction of a mucosal or a mucosal and systemic immune response that will protect the subject from a preselected pathogen or toxin. Purification of the time and temperature dependent conjugates can be achieved using standard protein purification methods that are well known in the art. For example, column purification methods can be used to isolated conjugated material away from the

unconjugated components. Examples of some suitable column resins include ion exchange resins, such as AG 50W-X1, X2, X4, X5, X8, X10, X12, and X16, which are available from Bio-Rad (Hercules, CA); gel filtration media such as Sephadex G-10, 15, 25, 50, 75, 100, 150 and 200, and Superdex 30, 75 and 200 prep grade, which are available from Amersham Pharmacia Biotech (Piscataway, NJ); and affinity chromatography media such as ligand presenting matrices, including ganglioside presenting matrices or toher carbohydrate presenting matrices, and antibody or antibody fragment presenting matrices. Also contemplated is hydrophobic interaction chromatography (HIC), which includes purification using phenyl sepharose, butyl sepharose, octyl sepharose, alkyl sepharose, which are available from Pharmacia Biotech (Piscataway, NJ).

Alternatively, the product can be used immediately after incubation without subsequent purification to immunize a subject requiring the induction of a mucosal or a mucosal and systemic immune response that will protect the subject from a preselected pathogen or toxin.

The present invention further contemplates the use of a solid phase matrix during the preparation of vaccine compositions. The solid phase matrix can be added to assist conjugation of the vaccine components or to assist in the purification of the components or the conjugated vaccine. Examples of suitable matrices include Gm1-affigel-10 matrix.

In an embodiment utilizing a solid phase matrix, the carrier component can be bound to a solid phase matrix, modified, exposed and conjugated to an immunogen, and then subsequently eluted from the matrix with a high salt solution, guanidinium thiocyanate, or other suitable elution buffer. Vaccine or vaccine components isolated in this manner can be subsequently dialyzed before use in vaccinations.

As discussed above, the present invention relates to the generation of vaccine compositions that facilitate the generation of mucosal and systemic immune responses. The vaccine compositions of the present invention comprise a carrier component and an immunogenic component. The carrier component of the vaccines of the present invention are contemplated to function by binding to the mucosa and exposing the immunogenic component of the vaccine composition to the mucosa. In one theory, the generation of the desired immune response by the vaccine compositions of the present invention occurs by increasing the exposure of the vaccine compositions to the target tissue. Any protein, peptide, or amino acid sequence that assists in the generation of a mucosal and systemic immune response by increasing the binding of the vaccine composition to the mucosa can be used in the present invention.

Further, the carrier components of the present invention can also possess adjuvant characteristics. Typically, adjuvants are used in vaccine compositions to enhance the immune response directed against the immunogenic component of the vaccine. The present invention further contemplates the use of carrier components that possess both mucosa binding characteristics and adjuvant characteristics. The present invention also contemplates using the time and temperature dependent methods of the present invention with carrier components with only adjuvant characteristics. Accordingly, the term carrier component is used by the present invention to

describe components that possess either mucosa binding properties, adjuvant properties, or both. Certain pathogenic toxins provide excellent carrier components. Examples of suitable toxins for use in the present invention comprise the cholera toxin, the shiga toxin, the staphylococcal α-hemolysin toxin, the staphylococcal δ-hemolysin toxin, the *Vibrio cholerae* thermostable direct hemolysin toxin, the pertussis toxin, and the *E. coli* heat-labile enterotoxin (Lt).

Subunits of these toxins can be used in the present invention. Suitable subunits include the A and/or B subunits of the cholera toxin, subunit A and/or B subunits of the *E. coli* heat-labile enterotoxin (Lt), *Bordetella pertussis* toxin subunits S2, S3, S4 and/or S5, the B fragment of diphtheria toxin, and the membrane binding subunits of shiga toxin or shiga-like toxins. Membrane binding portions of these toxins can also be used.

Other suitable subunits for use as carrier components in the present invention include *E. coli* fimbria K88, K99, 987P, F41, CFA/I, CFA/II (CS1, CS2 and/or CS3), CFA/IV (CS4, CS5 and/or CS6), P fimbraiae or other similar proteins. Other fimbriae contemplated within the scope of this invention include *Bordetella pertussis* filamentous hemagglutinin, *Vibrio cholerae* toxin-coregulate pilus (TCP), mannose-sensitive hemagglutinin (MSHA), fucose-sensitive hemagglutinin (PSHA), and the like.

Still other suitable subunits contemplated within the scope of the present invention include viral attachment proteins including influenza and Sendai virus hemagglutinins and animal lectins or lectin-like molecules including immunoglobulin molecules or fragments thereof, calcium-dependent (C-type) lectins, selectins, collectins or *Helix pomatia* hemagglutinin. Plant lectins with mucosa-binding subunits including concanavalin A, lipid A, pokeweed mitogen, wheat-germ agglutinin, phytohemagglutinin, abrin and ricin.

For all of the carrier components contemplated by the present invention, the whole molecule can be used as the carrier component, or a functionally active fragment of the molecule can be used. The present invention also contemplates the use of mutagenized forms of these molecules as carrier components.

In one embodiment of the present invention, the B subunit of the cholera toxin (hereinafter CtB) is used as the carrier component. It is well known in the art that vaccination of a subject with a target epitope in conjunction with CtB is an effective method of inducing mucosal immunity.

In one embodiment of the present invention, CtB is isolated, and purified for cross-linking to an isolated and purified toxin or toxoid (an attenuated toxin) that serves as the immunogenic component. In another embodiment, the isolated and purified CtB is cross-linked to an immunogenic component that is isolated either in part or as a whole pathogenic organism, such as a bacterium or a virus. The CtB source can be isolated from the bacterium itself or it can be produced using recombinant DNA techniques known in art. The CtB molecule used to form the vaccines of the present invention can be the whole protein, an immunogenic fragment of the whole CtB protein, a mutagenized form of CtB, or a fusion protein comprising the CtB protein or a fragment thereof and a suitable fusion partner. A suitable fusion partner for such a CtB fusion protein or a fragment thereof might be any protein, peptide or amino acid sequence which facilitates the expression and/or purification of the CtB fusion protein using recombinant DNA

techniques known in the art. Another suitable fusion partner or mutation for CtB might be any such protein or modification to CtB that facilitates cross-linking of the adjuvant to the immunogen to create bioactive conjugates. Bioactive conjugates are defined as coupled vaccine components possessing membrane binding affinity and target immunogenicity. The recombinant expression of CtB and CtB fusion proteins is disclosed in U.S. Patent Nos. 5,268,276 and 5,834,246, which are all hereby incorporated by reference.

The present invention is contemplated for use in any situation where a mucosal immune response or a mucosal combined with a systemic immune response to a toxin or pathogen is sought. Pathogens and toxins which gain entrance to the host organism through its mucous membranes are particularly suitable targets for the vaccines generated by the present invention. Such organisms cause a variety of conditions including sexually transmitted diseases, pulmonary, intestinal, lacrimal, and aural infections as wells as other disease conditions. Examples of such pathogens include: herpes viruses; human immunodeficiency virus (HIV); hepatitis B virus; *Mycobacterium leprae*; *Escherichia coli*; *Staphylococcus* spp., such as *S. aureus*; *Streptococcus* spp., such as *S. pneumoniae*, *Helicobacter pylori*; *Chlamydia trachomatis* (Chlamydia); *Neisseria gonorrhea* (gonorrhea); *Treponema pallidum* (syphilis); *Hemophilus ducreyi* (chancroid); and *Trichomonas vaginalis* (trichomoniasis). Protection against sexually transmitted cancer associated viruses such as certain strains of human papilloma virus are also contemplated as target pathogens. Other pathogens include: influenza viruses; rhinoviruses; *Mycobacterium tuberculosis* (tuberculosis); respiratory syncytial virus; rotavirus; *Corynebacterium diphtheriae* (diphtheria); *Bordetella pertussis* (pertussis); Japanese encephalitis; measles; rubella; mumps; and other pathogenic organisms that gain entrance to a host organism through its mucosal membranes.

Other examples of pathogens against which the present invention has utility are diseases that result in inflammatory pathology and which are known or believed to be caused by one or several opportunistic organisms. For example ulcerative colitis (e.g., *H. pylori*), colitis and other inflammatory bowel diseases, pelvic inflammatory diseases (e.g., *Chlamydia spp* and *Yrsinia spp.*), Reiter's syndrome, Lyme's disease, and any other chronic inflammatory conditions known or believed to be associated with an infectious organism.

Pathogens that create an immune response in a host upon infection are contemplated for use as the immunogenic component contained in the vaccine compositions of the present invention. Whole cells of the exemplary organisms listed above can be used (live, killed, or otherwise attenuated), or immunogenic portions thereof can be used. Such portions can be proteins, peptides, polypeptides, glycoproteins, lipids, or glycolipids.

The present invention is contemplated as having utility against additional diseases that can be reversed or modified by vaccination with the vaccine conjugates compositions of the present invention. Animal models for diseases such as diabetes, encephalomyelitis or arthritis have been shown to respond favorably to mucosal vaccination with rCtB-immunogenic conjugates, however, these conjugates were not prepared using the time and temperature dependent conjugation method of the present invention. (See, Sun et al., PNAS 93:7196 (1998); Bergerot et al., PNAS 94:4610 (1997); and Haque et al., Eur. J. Immunol., 26(11):2650-6 (1996)).

One aspect of the present invention envisions the use of an immunogenic component that is an epitope or immunogen known to or believed to produce an immunological response when encountered by a subject's immune system as an immunizing agent. The envisioned immunogen can be obtained from a natural source such as a bacterial or viral culture. Alternatively, the immunogen can be produced using recombinant DNA techniques known in the art. The entire immunogen can be produced, or immunogenic fragments thereof can be produced using a variety of techniques known to those skilled in the art.

In one embodiment of the present invention the diphtheria toxin can be used as the immunogenic component. In this embodiment, the diphtheria toxin can be isolated from a virulent form of *Corynebacterium diphtheriae* possessing a lysogenic β-phage which carries the diphtheria toxin gene. Alternatively, the toxin can be produced using recombinant DNA techniques known in the art. Such techniques can be used to produce the entire toxin from the toxin gene encoding the 62 kDa exotoxin or immunogenic fragments of the toxin can be produced. Once the diphtheria toxin is isolated, purified, and detoxified, it can be used in the method of the present invention to produce a suitable vaccine which increases both mucosal and systemic immunoglobulin production in response to vaccination with the vaccine. The detoxified form of the diphtheria toxin is known as the diphtheria toxoid (Dt).

The vaccines of the invention are formulated in several useful formats. All useful forms of the vaccines are contemplated by this invention. In one embodiment, the composition exists in the form of an atomized dispersion for use in delivery by inhalation. The atomized dispersion can be a solution containing the conjugated vaccine components. Typical carriers for atomized or aerosolized dispersions include buffered saline and other compounds well known to those of skill in the art. The delivery of the composition of the invention via inhalation has the effect of rapid dispersion to a large area of mucosal tissues, as well as absorption by the blood for circulation of the vaccine components.

The vaccines of the invention exist in the form of a suppository, whether rectal or vaginal. Typical carriers for formulation of the inactive portion of a suppository include polyethylene glycol, glycerine, cocoa butter and other compounds well known to those of skill in the art. Other suppository formulations suitable for delivery of the vaccines of the invention are also contemplated as part of the invention. Delivery of the vaccines of the invention via suppository has the effect of contacting a mucosal surface with the vaccines for release to proximal mucosal tissues. Distal mucosal tissues also receive the vaccines of the invention by diffusion.

Additionally, the invention contemplates the vaccine composition existing in the form of a liquid. The liquid can be for oral dosage, for ophthalmic or nasal dosage as drops, or for use as an enema or douche. When the vaccines of the invention exist in the form of a liquid, the liquid can be either a solution or a suspension of the vaccines. There are a variety of suitable formulations for the solution or suspension well known to those of skill in the art, depending on the intended use thereof.

Delivery of the vaccines of the invention in liquid form via oral dosage has the aim of exposing the mucosa of the gastrointestinal tract to the vaccines. A suitable dose, stabilized to resist the pH extremes of the stomach, would deliver the vaccines to all parts of the gastrointestinal tract, especially the upper portions thereof. The invention contemplates all means of stabilizing the vaccines in a liquid oral dosage such that the effective delivery of the composition is distributed along the gastrointestinal tract.

Delivery of the vaccines of the invention in liquid form via ophthalmic drops has the aim of exposing the mucosa of the eyes and associated tissues to the vaccines. A typical liquid carrier for eye drops is buffered and other compounds well known to those of skill in the art.

Delivery of the vaccines of the invention in liquid form via nasal drops has the aim of exposing the mucosa of the nose and sinuses and associated tissues to the vaccines. Liquid carriers for nasal drops are typically various forms of buffered saline.

Therefore, the vaccines of the invention are administered through a number of routes. Administration of the vaccines can be by nasal application, by inhalation, ophthalmically, orally, rectally, vaginally, or by any other mode that results in the vaccine contacting the mucosal tissues.

The present invention further contemplates a kit wherein the necessary components of a vaccine composition that elicit a mucosal and systemic immune response to a selected immunogenic component are found. In one embodiment of the present invention a suitable carrier component is provided. The carrier component can be pre-modified to induce cross-links with a desired immunogenic component, or it might be supplied in an unmodified state.

In one embodiment a cross-linking agent is enclosed in the kit. In one embodiment, the cross-linking agent is selected from the group comprising a variety of aldehydes, for example, glutaraldehyde, formaldehyde, glyceraldehyde, acetaldehyde, glyderaldehyde, phenylaldehyde, valeraldehyde, or 3,4-dihydroxyphenylacetaldehyde.

In another embodiment the kit contains compounds to stabilize the conjugated vaccine after incubation. Such stabilizing compounds include amino acids or other amine containing compounds added to quench or modify the cross-linking reaction.

In another embodiment the kit can be supplied with a portable method of purifying the combined, cross-linked vaccine after the cross-linking reaction has occurred.

The Examples described below discuss the methods used to generate the vaccines of the present invention and how they were used to generate a mucosal and systemic immune response to a desired pathogen or toxin.

EXAMPLE 1

Differential Effect of Various CtB Vaccine Preparations on the Production of Antibody Secreting Cells

To compare the effectiveness of various CtB based vaccines, four CtB-diphtheria toxoid (hereinafter Dt) conjugates were prepared. Recombinant cholera toxin subunit B (rCtB) was used in these experiments.

Preparation of rCtB-Dt Vaccines

Purified recombinant cholera toxin subunit B (rCtB) was conjugated to Dt to generate the vaccine compositions of the present invention.

Both the rCtB and Dt used to generate the vaccines were manufactured by SBL Vaccin AB (Stockholm, Sweden) under GMP manufacturing protocols and were provided as purified stable, non-toxic proteins in bulk liquid formulation. The diphtheria toxin was detoxified by the method of Neumueller, *Nature* 174:405 (1954), which is hereby incorporated by reference. The resulting diphtheria toxin was prepared by cultivating diphtheria bacteria in a semi-synthetic culture medium based upon acid hydrolyzed casein, amino acids and salts. The bacteria were then removed by sterile filtration. The toxin was then purified and detoxified.

An example of a common detoxification method is the method of Stainer, D.W., *Canadian J. Micro.*, 14:327-330 (1968), which is hereby incorporated by reference. Briefly, the Stainer method uses two methods to detoxify the diphtheria toxin. The first method detoxifies filtered, sterile toxin by the daily addition of between 0.1 to 0.15% formalin (a 37% solution of formaldehyde) for 4 days at a pH of 7.6 and subsequent incubation at 32°C for 35 days. This crude toxin was then concentrated by ultrafiltration and then purified by fractional precipitation with 25 and 43% ammonium sulfate. The 43% ammonium sulfate precipitate was dialyzed against 0.85% NaCl and sterilized by membrane filtration.

The second method of detoxification uses a sulfate-charcoal process before detoxification described by Pope & Stevens, *Brit. J. Exptl. Pathol.*, 39:139-149 (1958), which is hereby incorporated by reference. The purified toxin was diluted to approximately 500 Lf/ml and detoxified in the presence of 0.05 M L-lysine, 0.5% $NaHCO_3$, and 0.5% formalin as described by Linggood et al., *Brit. J. Exptl. Pathol.*, 44:177-188 (1963), which is hereby incorporated by reference.

Vaccine group C used in the following studies was generated using the time and temperature dependent method of the present invention. Vaccine group A was prepared using Dt coupled to rCtB with *N*-succinimidyl 3-[2-pyridyldithio]propionate (SPDP) using the method of Carlsson et al., *Biochemical J.*, 173: 723-737 (1978), which is hereby incorporated by reference. Vaccine group B was prepared using glutaraldehyde to couple the vaccine components using the method of Avrameas, & Ternynck, *Immunochemistry*, 8:1175-1179 (1971), which is hereby incorporated by reference. Vaccine group D was a commercial composition described below.

SPDP Conjugates (Vaccine A)

To create the SPDP conjugate, 42 mg/ml of rCtB and 37 mg/ml of Dt (quantified by ultraviolet light absorption (A_{280})) were separately subjected to G25 gel filtration in 0.1 M $NaHPO_4$, pH 8.5, and then separately concentrated to 12.8 mg/ml and 11.4 mg/ml respectively. Each protein was then modified with 4.6 µmoles SPDP for the rCtB and 2 µmole SPDP for the Dt, both for thirty (30) minutes, at 22°C, creating 2-pyridyl disulfide moieties at the proteins amino groups. The rCtB sample was then subjected to G25 gel filtration in 0.1M $NaHPO_4$, pH 7.5 and recovered in a volume of 7 ml. The derivatized Dt was reduced with 100 µl of 1M dithiothreitol (DTT) for thirty (30)

minutes at 22°C. The reduced Dt derivative was then subjected to G25 gel filtration in a medium of 0.1 M $NaHPO_4$, pH 7.5 and recovered in a volume of 7 ml.

Equal volumes of the two proteins (7 ml) were mixed together and incubated overnight at 22°C to allow disulfide exchange to occur between the thiol moiety of Dt and disulfide groups on rCtB. The resulting product was a stable conjugate of mixed disulfide linkage between rCtB and Dt. The solution was then concentrated to 2.5 ml. The solution was subjected to G25 gel filtration in PBS and recovered in a volume of 4 ml. The filtrate was stored at 4°C for 3 weeks. A fraction of this product was taken for chromatographic/antigenic profile analysis.

After incubation, preparative gel filtration analysis was performed on the rCtB-Dt mixture. Three runs over a size exclusion Superdex 200 PG (Prep Grade) High Load 26/60 FPLC column in phosphate buffered saline, pH 7.4 were performed. Eluate fractions were analyzed by Gm-1 ELISA for diphtheria antigen with a horse anti-Dt antiserum and positive activity fractions 21-33 were pooled and concentrated to 4 mg/ml. The pooled proteins were sterile filtered, aliquoted and stored at 4°C for use in vaccinations.

Glutaraldehyde conjugates (Vaccine B)

Similarly, glutaraldehyde conjugates of rCtB and Dt were prepared using 22 mg of rCtB and 19 mg of Dt, both of which were quantified by UV A_{280} absorption. Each protein solution was subjected to gel filtration on G25 in 0.1 M sodium carbonate, pH 9.5. The proteins were then concentrated to 16.7 and 14.6 mg/ml respectively. The Dt was modified with glutaraldehyde (4% final concentration) for forty-five (45) minutes at 22°C. The modified Dt was subjected to G25 gel filtration in a medium of 0.1M $NaCO_3$, pH 9.5 and recovered in a total volume of 3.5 ml.

Portions of each solution (1.1 ml of rCtB and 3.5 ml of Dt) were then mixed and stored overnight at 22°C. The conjugation reaction was then quenched with 0.5 ml of 1M ethanolamine for thirty (30) minutes at 22°C. The solution was concentrated to 2 ml and subjected to G25 gel filtration in a PBS (phosphate buffered saline) medium and recovered in a 4 ml volume which was stored for 3 weeks at 4°C. A sample was taken for chromatographic/antigenic profile analysis.

The mixed proteins were then subjected to preparative gel filtration purification in PBS. From three separate runs fractions 21-29 were pooled to form Pool I which was concentrated to 4 mg/ml, sterile filtered, aliquoted and stored at 4°C. Pool I was chosen for use in the animal studies. Fractions 30-35 were pooled to form Pool II, sterile filtered, aliquoted and stored at 4°C. Analytical gel filtration, Gm1 ELISA, and SDS-PAGE polyacrylamide gel electrophoresis was performed on both samples. An example of the gel filtration and ELISA results is found in FIGURE 1.

FIGURE 1 shows an overlay plot of protein content (A_{280}) and Gm-1 bound Dt antigenic profiles for each conjugate after fractionation by gel filtration. Gm-1 binding is expected to increase the vaccine's potency through delivery to M cell gangliosides and subsequent processing by the mucosal immune system. Finally, the Gm-1 binding

fractions positive for Dt antigen by the Gm-1 ELISA were pooled for vaccination. The ELISA methodology is described in detail below.

The SPDP and glutaraldehyde conjugated vaccines each contained approximately 50% diphtheria toxoid by mass. The protein concentration at the anti-diphtheria antigenic endpoint titer (defined as background + 0.4 O.D. units by ELISA) was determined for each vaccine preparation. The minimal Gm1 antigenic concentration bound of the vaccines were 15.1 μg/L (SPDP) and 12.5 μg/L (glutaraldehyde) indicating that the diphtheria antigen could be detected in as little as 750 picograms (50 μl assay aliquots) of vaccine.

Embodiment of the Present Invention (Vaccine C)

Animals from this test group were immunized with a time and temperature conjugated vaccine composed of Dt and rCtB. For this study a formaldehyde treated diphtheria toxoid was mixed with cholera toxin B subunit at a final concentrations of 4.6 mg/ml Dt and 1.4 mg/ml rCtB. The mixture was prepared and a sample was used immediately for the initial immunizations. The remaining mixture was incubated at 4°C for use in subsequent immunizations. Subsequent immunizations occurred at two weeks and four weeks. Thus, the mixture of vaccine C used in the booster rounds of immunization was incubated at 4°C for two and four weeks.

At the times of immunization, mice from this test group were immunized with 35 μl of the vaccine mix by the intranasal method described below.

DUPLEX Vaccine (Vaccine D)

Vaccine D was purchased from SBL Vaccin AB (Sweden). It is a commercially available vaccine containing both diphtheria toxoid and tetanus toxoid adsorbed onto alum and suspended in solution. Composition of the vaccine includes 30 Lf diphtheria toxoid, 7.5 Lf tetanus toxoid, 5 mg alum, 0.1 mg thimerisol (a preservative), all diluted into 1 ml phosphate buffer.

ELISA METHODOLOGY

The ELISA method used in these studies was developed to monitor rCtB and was based on the published methods of Svennerholm & Holmgren, *Curr. Microbiol.*, 1:19-27 (1978) and Holmgren, *Infect. Immun.*, 8:851-859 (1973), which are both hereby incorporated by reference.

A GM1 ELISA assay was used to detect diphtheria toxoid bound to rCtB. In this assay an ELISA plate (typically a 96-well plate known to one skilled in the art), was coated with ganglioside Gm1 at a concentration of 0.3 nmol/ml in PBS. 100 μl/well of the Gm1 solution was applied to the wells of the plate overnight at 4°C. The plates were then washed with PBS to remove any unbound Gm1. The plates were then blocked with 0.1% bovine serum albumin in 200 μl PBS at /well at 37°C for sixty minutes. The blocking solution was then removed and 50 μl of a 5 μg/ml conjugated protein solution was added to the wells. Serial dilutions of 1:3 were performed. The samples were

then incubated on the plates for seventy-five (75) minutes at room temperature. Following this incubation, the plates were washed with a PBS/0.05% Tween 20 solution.

After the plates were washed, the primary antibody was applied. In this example horse anti-diphtheria toxoid serum (SBL Vaccin AB; Stockholm, Sweden) was applied at a dilution of 1/2000 in PBS containing 0.1% BSA, 0.05% Tween 20 at 100 μl per well for ninety (90) minutes at room temperature. The plates were then washed with the PBS/0.05% Tween 20 solution. Following the wash, a secondary antibody was applied to the plate. Here, the secondary antibody was a horse radish peroxidase conjugated goat anti-horse Ig antibody. The secondary antibody was diluted at 1/2000 in PBS containing 0.1% BSA, 0.05% Tween 20 and applied at 100 μl per well for seventy-five minutes at room temperature. The plates were then washed with PBS/0.05% Tween 20.

Color from the secondary antibody was developed by adding the horseradish peroxidase substrate ortho-phenylene diamine (OPD) to the plates in a solution containing the following components: o-phenylenediamine 10 mg, 6 ml 1M citric acid, 12.5 ml of water, 6.4 ml 0.2 M Na_2HPO_4, and 10 ml H_2O_2. After the color developed, the reaction was quenched with 25 μl of 3M HCl. The plates were then read in a spectrophotometer at 492 nm.

Vaccination of Mice with rCtB-Dt Vaccine Formulations

Large doses of antigen were initially chosen for vaccinations to maximize the immunogenic response to the vaccine formulations. In previous studies, 100 μg of intranasal conjugate clearly generated elevated levels of both IgG and IgA in serum and mucosal tissues of mice (data not shown). In the present study, groups of ten balb/c mice were immunized with vaccines. Each mouse received 100 μg total protein intranasally three times biweekly. Inoculates were given in 25 μl of PBS with either vaccine A (SPDP), vaccine B (glutaraldehyde) conjugates or 35 μl of vaccine C (a time dependent conjugation of rCtB and Dt). Intranasal immunization was performed under mild anesthesia using isoflurane. When the mice were sufficiently anesthetized, a pipette was used to place the vaccinating dose near the nostrils of the animals so the mice would inhale small droplets of the vaccine. Within 2-3 normal breathes, the injectate was inhaled. The mice remained anesthetized for 1 minute total and remained on their backs until they awoke.

One additional group of mice received 100 μl of DUPLEX (vaccine D; containing 3 limit flocculation units (Lf) Dt + 0.75 Lf Tt) three times intraperitoneally into either the lower left or right quadrant of the animal. Only moderate pressure was used. All animals were bled once prior to immunization and 10 days following each vaccination. Half of the animals were killed 8 days following their last immunization. Their lungs and spleen were minced into single cell suspensions and analyzed by ELISPOT according to the method of Czerkinsky et al., *J. Immuno. Methods*, 65:109 (1983), which is hereby incorporated by reference.

Freshly isolated spleen and lungs were teased into single cell suspensions in RPMI 1640 supplemented with 5% FCS and 1 mM L-glutamine (complete medium) for ELISPOT analysis according to the method of Czerkinsky et al., *J. Immuno. Methods*, 115:31-37 (1988), which is hereby incorporated by reference. Cells were washed three times

and counted in a hemocytometer. Cells were then resuspended to 2 x 10^6 cells/ml and 200 μl transferred to a 96 well micro-culture plate. Four serial dilutions of 1/3 were performed in complete medium. Each cell titration was then transferred to an ELISPOT plate (Millipore Corp.; Bedford, MA), previously coated with 5 μg/ml Dt or Gm-1 + rCtB and blocked with RPMI and 5% fetal calf serum (FCS). ELISPOT plated cells were placed in a vibration free, 5% CO_2 humidified incubator overnight. The plates were then washed and incubated with 50 μl HRP-labeled goat anti-mouse IgA plus alkaline phosphatase-labeled goat anti-mouse IgG for 1 hour at room temperature. After a final wash, each plate was developed sequentially for two colors with BCIP/NBT solutions (Bio-Rad; Hercules, CA) followed by AEC according to manufacturers protocols. The reactions formed red spots where antigen specific IgA was secreted or blue/black spots where antigen specific IgG was secreted. The number of IgG or IgA secreting cells with antigen specificity were then counted in a dissecting microscope.

The remaining animals were killed one month following the last immunization and all sera, cells, tissues and fluids were retained in appropriate storage conditions. All animals were perfused with 0.1% heparin in sterile PBS and one lung was placed in a tube containing 200 μl PBS with 100 μg/ml soybean trypsin inhibitor, 350 μg/ml phenylmethylsulfonylfluoride (PMSF), 0.1 M EDTA and 0.1% BSA (Sigma-Aldrich Co.; St. Louis, MO). Tissue fluids were stored at -80°C until ELISA analyses of their anti-Dt and rCtB antibody titers described below. The second lung and spleen were placed in RPMI 1640 with 5% fetal calf serum and 1 mM L-glutamine (Biowhittaker, Inc.; Walkersville, MD) on ice for ELISPOT processing described above.

Antibody Secreting Cells in the Lungs and Spleen

We have determined that the upper respiratory tract was immunized with the experimental vaccines by ELISPOT analysis discussed above. The assay was used to identify the number of Dt specific IgG and IgA antibody secreting cells (ASCs) in the lungs and spleen of immunized animals eight days after the last immunization. It is known that by eight days following immunization, B cells have migrated to the mucosal tissues.

Shown in FIGURE 2 are the relative number of antibody secreting cells that have infiltrated into the lungs and spleen of vaccinated animals. Group mean numbers of anti-Dt IgG spleen cells are shown in panel A and anti-Dt lung cells are shown in panel B. IgG antibody secreting cells (ASCs) are shown with dark bars while the IgA ASCs are shown with open bars. Animals in vaccine group A received intranasal SPDP conjugate, group B received intranasal glutaraldehyde conjugate, group C received the time and temperature conjugated intranasal vaccine of the present invention and group D received intraperitoneal DUPLEX vaccine.

As shown in FIGURE 2, greater numbers of Dt specific ASCs were found in the lungs of animals immunized with vaccines A & C than with vaccines B or D (FIGURE 2. The group geometric mean for animals receiving vaccine A was 455±100 anti-Dt IgG secreting cells and 13±10 IgA secreting cells in the lungs, while the lungs of animals receiving vaccine C had 253±250 IgG secreting cells and 65±63 IgA secreting cells of 10^6 total cells. Animals receiving vaccine B had only 34±33 IgG secreting cells and 2±1 IgA secreting cells in 10^6 cells on average. In

contrast, only one animal receiving vaccine D was found with IgG anti-Dt secreting cells in its lungs and none of the animals had IgA anti-Dt secreting cells. These data suggest that a significant mucosal response to Dt in the lungs was induced by intranasal vaccination with either vaccine A or C as evidenced by the infiltration of the lungs with anti-Dt antibody secreting cells.

FIGURE 2 also shows that the numbers of antigen specific ASCs found in the spleen were lower than those observed in the lungs. The largest numbers of anti-Dt IgG secreting cells in the spleen were found in animals immunized with vaccine D (DUPLEX), as expected. However, relatively small numerical differences in the IgG ASCs were found in the spleen between vaccines. While vaccine D induced 156±33 ASCs /10^6 cells, vaccines A, B & C, respectively induced 21±9, 5±4 and 47±39 IgG ASCs. The number of IgA ASCs was still lower. Vaccine C induced 15±9 IgA ASCs in the spleen. The conventional vaccine D was the next most potent vaccine with only 2±1 IgA ASCs / 10^6 cells.

It is known that the spleen is a major repository for systemically activated ASCs. Yet, experiences in generating hybridomas teaches that more dramatic numbers of cells are often observed around three days following systemic immunization rather than eight days as used in the present study. Therefore, it is possible that the splenocyte numbers obtained eight days after immunization represent a declining number of localized cells. Nonetheless, given the known potency of the DUPLEX (group D) vaccine for inducing protective antibody, this data appears to reflect a systemic response to the intranasal vaccines of the present invention which is comparable to that induced by commercial diphtheria vaccines.

Serum and Mucosal Tissue Antibody Responses

Antibody responses to the conjugates were confirmed by quantitative ELISA according to the method of Engvall & Perlman, *Immunochemistry* 8:871-874 (1971), which is hereby incorporated by reference. All titers are reported as reciprocals of the lowest dilution which generated an optical density of 0.4 over background. Briefly, antigen specific antibodies are determined by ELISA assay according to the methods of Engvall & Perlman (*Id.*) and Handley et al., *J. Immuno. Methods* 54:291-296 (1982), which is hereby incorporated by reference. ELISAs were performed with 96 well A/2 microtiter plates (Corning Costar; Acton, MA) coated with 50 μl of 5 μg/ml of diphtheria toxoid (SBL Vaccin AB; Sweden) or Gm-1 (Sigma-Aldrich Co.; St. Louis, MO) for 1 hour at 37°C. Gm-1 plates were washed and 50 μl of a 2.5 μg/ml rCtB (SBL Vaccin AB) was added. Unbound antigenic material was removed and the plates blocked with 0.1% bovine serum albumin (Sigma-Aldrich Co.; St. Louis, MO) in PBS under the same conditions. The blocking solution was then removed and the plates were washed with 0.05% Tween-20 (Sigma-Aldrich Co.; St. Louis, MO) in PBS. Next, 50 μl of a four fold serial dilution of serum or tissue fluids were incubated in 0.1% BSA and 0.05% Tween 20 in PBS for 1 hour at 37°C. Plates were washed again as described above followed by the addition of 50 μl of a 1/3000 dilution of horseradish peroxidase (HRP)-labeled goat antisera to mouse IgG or IgA (Jackson ImmunoResearch Labs, Inc.; West Grove, PA) to each well for an additional hour at 37°C. The plates were then washed again to remove unbound antibody and 50 μl of OPD (Sigma-Aldrich Co.; St. Louis, MO) was added for 10

minutes before the reaction was stopped with 25 μl 3M HCl solution. The absorbance of each well was read on a SpectroMax 250 spectrophotometer (Molecular Devices Corp.; Sunnyvale , CA) at 492 nm.

Tissue fluids were extracted from organs that had been prepared in the following manner. Each mouse was perfused with 0.1% heparin and the appropriate organs were excised, mixed with a cocktail of protease inhibitors, and frozen at -80°C for 24 hours. The organs were thawed, saponin was added to 2%, and the samples were then frozen again for an additional 24 hours at -80°C. The samples were then thawed at 4°C overnight. The fluid bathing the organs was collected and assayed for Dt specific immunoglobulin by ELISA.

Tissue immunoglobulins generated by each of the vaccines are shown in FIGURE 3. Vaccine C induced titers of IgG anti-Dt in excess of 90,000 and IgA anti-Dt titers in excess of 200 in the lungs of vaccinated animals. In contrast, vaccines of SPDP (A) and glutaraldehyde (B) induced titers of 5,000 and 4,000, respectively and commerical vaccine (D) induced a titer of 17,000. None of these vaccines were observed to induce large titers of IgG or IgA to Dt in the distal mucosal tissues of the small intestines. Thus, vaccine C seemed more potent than other vaccines for the induction of mucosal immunity at or near (lungs) the site of vaccination.

Endpoint titers can vary in part because of changes in IgG concentration. Therefore, the IgG and IgA concentrations for the fluids obtained were prior to and 10 days following the final vaccination. Changes in total immunoglobulin concentrations can also be an indication of the adjuvant or non-specific stimulation of the mucosal immune response.

The relative amounts of IgG and IgA retrieved from the lungs of vaccine C recipients (53.61 and 7.66 μg, respectively) were determined and found to be clearly elevated over those found in recipients of vaccines A, B, or D (6.06 and 1.13 μg; 8.13 and 2.18 μg; 20.91 and 0.38 μg). Serum immunoglobulins increased as well. Vaccine C induced a change in IgG concentration from 0.8 mg/ml to 81 mg/ml. Vaccine D has a comparable effect, elevating IgG from 0.8 mg/ml to 53 mg/ml. In contrast, vaccines A and B had a less dramatic influence on total IgG concentration, causing only 3-4 fold increases in total IgG concentration in the serum. Similarly, serum IgA concentrations increased from ≈40 ng/ml to 112.2; 130.3; 146.32 and 95.4 ng/ml with vaccines A, B, C and D, respectively. Each of the nasal vaccines appeared to have an additional influence on serum IgA over that observed with systemic vaccination. Therefore, mucosal vaccination with rCtB-Dt conjugate seemed to have a more dramatic adjuvant effect on the total immunoglobulin concentrations in both the tissue and the serum than did systemic immunization. This adjuvant effect seemed greatest with vaccine C.

FIGURE 4 shows the pooled serum IgG anti-Dt titers for the vaccination groups of 10 animals during the vaccination protocol. All vaccinated animals responded to Dt with high titer serum IgG antibody eight days (FIGURE 4, 5 weeks) following their final vaccination. Vaccine C induced IgG anti-Dt titers (~900,000) comparable to titers from commercial vaccine D (~500,000) despite its different route of administration. All serum antibody titers remained elevated over pre-immune controls four weeks following the final vaccination (FIGURE 4, 8 weeks). Little change in titer from vaccines A or B were noted, while vaccine C dropped to ~400,000 and vaccine D dropped to >300,000.

Thus, mucosal vaccination by the intranasal route with the present invention generated large increases in circulating serum antibody in addition to site specific mucosal antibody production.

The unexpected results of this study indicate that vaccine C is equal to or more effective than the commercial vaccine and the other methods of vaccine component conjugation. Vaccine C is shown to be more effective in both the recruitment of IgG and IgA ASCs to the lungs and spleen and in the induction of increased levels of IgG and IgA in the serum. Further, vaccine C produced large increases in lung fluid IgG and IgA against Dt. The data also show that both vaccines A and C can induce mucosal and systemic immune responses to diphtheria toxin when administered by the intranasal route. We anticipate that these high titre antibody containing fluids represent protective or toxin neutralizing antibody titers in the serum and lungs of the immunized animals.

The results from vaccine C were unexpected since vaccine C was composed of a mixture of vaccine components without the further addition of a conjugating agent. Thus it should have produced only a minimal antigenic response from those animals that were immunized with the mixture. Since the mixture produced a mucosal and systemic immune response, some method of conjugation of the vaccine components was suspected to have occurred.

It was observed that the Dt vaccine component used in this study had been detoxified by treatment with formaldehyde. The uncoupled vaccine components in vaccine C became conjugated over time during the incubation at 4°C. This conjugation, which occurred over time produced a superior vaccine product that resulted in the induction of a superior immune response from those animals immunized with vaccine C as compared to those immunized with the other vaccine components.

Parameters of rCtB Conjugated Vaccine Compositions

The results from Example 1 showed that rCtB vaccine compositions conjugated in a time and temperature dependent manner provided superior immunogenic responses when compared to other rCtB vaccines. To investigate the parameters of the above described phenomenon, further experiments were conducted on the time and temperature conjugated rCtB-diphtheria toxoid vaccine used in Experiment 1 using a Gm1 ELISA assay.

EXAMPLE 2

The Effect of Time on Antigenicity of Vaccine Conjugate Formation Conjugations

The amount of rCtB and Dt conjugation occurring in Vaccine C of Example 1 was examined using the Gm1 ELISA assay. Those assays measure the extent of CtB binding to Gm1 bound to the assay plate using antibodies. The procedure for this assay is discussed in Example 1. Briefly, a microtiter assay plate was prepared by applying and binding Gm1 to the wells of the plate. Subsequently a CtB containing vaccine was applied to the assay plate to determine the extent of immunogen binding to the CtB carrier component. After washing the unbound vaccine from the plate, a primary antibody specific for CtB was applied. This antibody would bind to any CtB in the wells of the

assay plate. After washing the unbound antibody from the plate, a secondary antibody was then added. The secondary antibody carried an enzyme which produced a quantitative signal that indicated the presence of the primary antibody when a colorimetric substrate was introduced to the plate.

This assay was also used to detect the presence of Dt bound to CtB. As described above, a CtB containing vaccine was applied to an ELISA assay plate prepared with Gm1. The applied CtB bound to the Gm1 molecules on the assay plate. In contrast to the previous method, a primary antibody specific for Dt was applied to the wells of the assay and that antibody was bound to any Dt on the plate. A similar signal producing secondary antibody was used in this experiment to produce a signal to measure the amount of Dt present in the plate. Since Dt does not bind to Gm1, the Dt must be conjugated to CtB bound to the plate. Given this specificity, this assay provides a method to determine the amount of conjugation occurring between the CtB carrier component and the Dt immunogen molecules using the time and temperature dependent conjugation method of the present invention.

In Example 2, the amount of conjugation in freshly mixed rCtB and Dt vaccine components was compared to that of the time and the temperature conjugated vaccine from Example 1. The amount of conjugation is defined here as the ability of the vaccine mixture to present the immunogen on a Gm1 surface and is measured as an antigenic titre and presented in terms of relative ELISA units (EU). In this experiment a fresh mixture of rCtB and Dt toxoid was created and analyzed in the Gm1 ELISA assay. These results were compared to the ability of the time and temperature incubated vaccine C used in Experiment 1 to produce a signal in the Gm1 ELISA assay.

Both the fresh admix and the conjugated vaccine preparation (CVP) used in Experiment 1 were produced using similar methods. Briefly, both vaccines were prepared by mixing diphtheria toxoid and rCtB together to final concentrations of 4.6 mg/ml and 1.4 mg/ml, respectively. By the time of this experiment, the CVP (vaccine C) had incubated at 4°C for 64 days. The fresh admix was prepared immediately before application to the Gm1 ELISA assay plate.

FIGURE 5 shows the anti-CtB and anti-Dt responses of the two vaccine preparations. The results show that only the CVP possessed anti-Dt activity, an indication of carrier component-immunogeninc component conjugation. As a control rCtB and Dt alone were tested for their ability to bind to the assay plate. Binding of rCtB was detected in all the samples containing that protein, (rCtB alone, the fresh admix and the aged vaccine C). No response was detected from Dt alone. When the freshly mixed vaccine assay wells were probed for Dt binding, no response was detected, even though rCtB binding was shown. However, when the CVP (vaccine C) was probed for Dt binding, a relatively strong signal was detected.

These results indicate that it is the combination and incubation of the two vaccine components over time which produced a conjugation of the vaccine components. This conjugation can be responsible for creating a vaccine which presents the immunogen to the immune system of the immunized animals in a superior manner compared to other methods.

To further investigate the question of immunogen presentation, the other vaccines used in Experiment 1 were examined for their ability to present the Dt antigen in the Gm1 ELISA assay.

FIGURE 6 compares the effectiveness of the conjugated vaccines A, B, and C to present the Dt immunogen in the Gm1 ELISA assay. At the time of testing all of the vaccines had been aged for 64 days at 4°C.

The positive control rCtB alone, displayed a significant signal when analyzed in the ELISA assay. The units of measurement are reported as reciprocals of the lowest dilution which generated an optical density of 0.4 over background. The aged rCtB-Dt vaccine C sample possessed a relative Dt antigenic titer of 83 EU while the SPDP and glutaraldehyde fixed vaccines produced smaller antigenic titers. This figure illustrates the superiority of the conjugation method of the present invention since the time and temperature dependent conjugation of the vaccine components appears to produce a superior antigen.

EXAMPLE 3

The Effect of Time on Vaccine Conjugate Formation

The time course over which rCtB-Dt conjugates appear was examined in this experiment. Formaldehyde detoxified diphtheria (rCtB-Dt) and tetanus (rCtB-Tt) vaccines were prepared using the time and temperature incubation method of the present invention over a range of immunogen to carrier ratios. Solutions of diphtheria toxoid (5.2 mg/ml) or tetanus toxoid (2.2 mg/ml) were mixed with rCtB (11.8 mg/ml) in ratios of 0.3:1, 1:1, 3:1, 6:1, or 9:1 by mass. Each solution was incubated at 4°C. Samples were taken at 2 and 4 weeks, diluted to 5 μg/ml in PBS and analyzed in the Gm1 ELISA assay for toxoid presentation. The tetanus toxoid Gm1 ELISA assay was performed in the same manner as for the diphtheria toxoid in the previous experiments except that horse anti-tetanus toxoid antiserum was used at 1/2000 dilution in place of horse anti-diphtheria toxoid antiserum.

FIGURE 7 compares the ability of the two vaccine compositions to display the immunogen component of the vaccine as a function of time and ratio of toxoid to rCtB. A fresh mixture of either Dt or Tt and CtB failed to produce any Dt or Tt binding to the Gm1 plate, indicating no conjugation. A clearly superior ability of the rCtB-Dt vaccine combination sampled at 4 weeks to present the Dt epitope in the Gm1 ELISA was observed in this figure as compared to that of the 2 week sample.

The results of this experiment show again that the method of the present invention to conjugate carriers and immunogens improves with time of incubation. It also shows the optimal ratios of toxoid to carrier above which antigenicity fails to appreciably increase. The data also shows that tetanus toxoid binding changed significantly from 0 to 14 days. By the 14th day this preparation appeared to come to equilibrium. It is likely the subtle differences in detoxification methods or in formaldehyde treatment of each immunogen may have caused the observed differences in conjugation profile. Further, an optimal ratio of rCtB:Dt of 1:3 was observed for this particular combination.

EXAMPLE 4

Time Course of Conjugation

To further examine the temporal component of the time and temperature dependent conjugation method of the present invention, Dt and CtB were mixed and incubated. Diphtheria toxoid from two commercial sources were examined for time dependent conjugation (SBL Vaccin AB; Sweden and Staten Serum Institute (SSI); Copenhagen, Denmark). In the sample containing Staten Serum Institute Dt, the Dt and CtB were mixed together to give final concentrations of 3.9 mg/ml and 1.3 mg/ml, respectively. In the sample containing the SBL Vaccine Dt, Dt and CtB were mixed together at the final concentrations of 4.5 mg/ml and 1.5 mg/ml, respectively. The conjugation reactions were prepared and incubated for 0, 1, 2, or 4 weeks at 4°C as indicated in FIGURE 8. Following the specified incubation period, a portion of each sample was collected and frozen at -20°C for future analysis. After all samples had been collected, they were thawed at room temperature and submitted to Gm1 ELISA with detection for Dt as described in Example 1.

The time dependence of the conjugation method of the present invention for the two commercial Dt preparations is shown in FIGURE 8. The amount of conjugate of diphtheria toxoid and rCtB steadily increases during the first 4 weeks of incubation.

EXAMPLE 5

The Effect of Temperature on Vaccine Conjugations and GM1 ELISA Assay

In this Example rCtB-Dt conjugates were formed at various temperatures to determine an optimum temperature of conjugation. Dt and rCtB were mixed to give a final solution of 4.6 mg/ml and 1.3 mg/ml respectively. Portions of this master solution were placed at 4°, 22°, 37°, 45° and 70°C. After one month, a sample of each solution was diluted in PBS to 5 μg/ml and assayed in the Gm1 ELISA assay. The presence of rCtB and Dt was detected in parallel wells to determine the extent of immunogen presentation.

FIGURE 9 shows the results of this experiment. A clear trend in both carrier component and immunogenic component presentation appears over the range of incubation temperatures. Although little difference in Dt antigenicity occurred between the 4° and 22°C temperatures of incubation, rCtB presentation appeared diminished at 22°C. The antigenicity of both vaccine components dropped significantly when incubated at 37°C. These reduced signals were also observed at 45°C and 70°C.

The results of this experiment suggest that an optimum temperature of incubation is at or below 22°C. This observation is likely the result of the reported instability of CtB at higher temperatures.

EXAMPLE 6

pH Optimum for Conjugation

A series of time and temperature dependent conjugations of Dt and rCtB were performed at different pH values to determine the pH optimum of conjugation. A mixture of Dt (5 mg) and rCtB (5 mg) was prepared to final concentrations of 1.2 mg/ml Dt and 1.2 mg/ml rCtB. Aliquots (0.7 ml) of this solution were applied to G25 gel

filtration columns (PD10; Pharmacia, UK) equilibrated with one of the following buffers: 50 mM sodium acetate, pH 5.0; 50 mM sodium phosphate, pH 6.0; 50 mM sodium phosphate, pH 7.0; 50 mM sodium phosphate, pH 8.0; 50 mM sodium carbonate, pH 9.0; or 50 mM sodium carbonate, pH 10.0. The protein was eluted from the columns in a collected volume of 3.5 ml, and each solution was concentrated by centrifugation in a Centricon 30 (Millipore Corp.; Bedford, MA) to 1 ml. The reactions were stored at 4°C for two weeks and then assayed for Dt-CtB conjugates by Gm1 ELISA.

The results, shown in FIGURE 10, indicate that the optimum pH for conjugation of Dt and rCtB is near pH 8.0. Some protein precipitated from solution after buffer exchange into the solutions with pH 5, 6, and 7. It appeared that at pH 5 and 6 the proteins precipitated almost completely, whereas at pH 7 only some precipitate was visible. It has been reported that cholera toxin subunit B undergoes pH-dependent conformation changes with transitions near pH 5, which include dissociation of the pentamer into monomers. (Ruddock et al., *Biochemistry*, 35: 16069-16076 (1996)) Diphtheria toxin has also been reported to undergo a pH-induced conformational transition that involves a massive unfolding of the toxin molecule. (Ramsay et al., *Biochemistry*, 28: 529-533 (1989)). This process is centered at pH values between 4.7 and 5.4. The protein precipitation reported here can be related to these conformational transitions.

EXAMPLE 7
IgG cross-linking to rCtB with Formaldehyde

Horse IgG was treated with formaldehyde and amino acids under various conditions to determine the most effective method of preparing conjugates between rCtB and formaldehyde treated proteins. The conditions studied were similar to those reported in Neumueller, *Nature*, 174:405 (1954), for the detoxification of diphtheria toxin.

Horse IgG was dissolved to a concentration of 1 mg/ml in 50 mM sodium phosphate, pH 7.5, in the presence or absence of 0.1 M glycine. Formaldehyde was added to a final concentration of 0.1%, and the solution incubated at 35°C for 8 days (absence of glycine) or 25 days (presence of glycine). After incubation, excess formaldehyde was removed from the solution by exhaustive dialysis against 0.1 M sodium phosphate, pH 7.5. Then 1 mg of cholera toxin B was added (in 1 ml PBS) to 1 mg of IgG. As indicated in the figure legends, 1 M glycine in 0.1 M sodium phosphate, pH 7.5, was also added to some reactions to a final concentration of 0.1 M glycine. Each sample was concentrated in a Centricon 30 microfiltration device from Millipore Corp. (Bedford, MA) to a volume of 200 ml. The samples were then incubated at 4°C for several weeks.

Following incubation, the conjugates were analyzed. The protein concentration of each sample was determined by Bio-Rad protein assay (Bio-Rad; Hercules, CA). The relative amount of horse IgG conjugated to rCtB was determined by a Gm1 ELISA binding assay. The Gm1 ELISA protocol used is similar to that described for measuring diphtheria-cholera toxin B conjugates. Briefly, Gm1 coated 96-well plates were prepared and blocked with 0.1% bovine serum albumin. A solution of 5 µg/ml conjugated protein was added to the wells of one row and a serial dilution of 1:3 was performed. The samples were incubated on the plates for 75 minutes at room temperature. Following the incubation, the plates were washed with PBS/0.05% Tween 20. After the plates were washed, a

horseradish peroxidase conjugated goat anti-horse IgG antibody was applied at a dilution of 1/2000 in 0.1% BSA/0.05% Tween 20, 100 ml per well. The plates were incubated for 75 minutes at room temperature. After this incubation, the plates were washed with PBS/0.05% Tween 20. The plate was developed with ortho-phenylene diamine (OPD) as previously described in Example 1. The results are reported in relative ELISA Units (EU) in FIGURE 11.

The results shown in FIGURE 11 represent the detection of conjugates of IgG to rCtB shown as the relative amount of IgG (Elisa Units) able to bind to Gm1 in a Gm1 ELISA assay. To account for any loss of material during the procedure, the protein content of each sample was measured with a Bio-Rad (Hercules, CA) protein assay and used to normalize the ELISA results. As seen from samples a and b, inclusion of the amino acids during the formaldehyde (FA) modification of the horse IgG procedure greatly reduced the amount of IgG-CtB conjugate bound to Gm1. In contrast, samples produced by FA modification of IgG in the absence of amino acids (sample c) or by the addition of glycine after the addition of rCtB (sample e) showed significantly more Gm1 binding activity. The concomitant addition of the amino acid glycine with the addition of rCtB (sample d) showed a reduce amount of Gm1 binding as compared to samples c and e, however, the Gm1 binding of sample d was significantly higher than that of either samples a or b, where the samples were produced with the addition of glycine or lysine during the FA modification of the IgG. It should be noted that Vaccine C was prepared in a manner analogous to the preparation A in FIGURE 11. Therefore, more immunogenic product is anticipated from preparation E.

The results of this example show that, in general, treating IgG with formaldehyde without the addition of other amino acids produces higher levels of rCtB conjugation. Inclusion of amino acids during formaldehyde modification led to approximately 10-fold lower levels of conjugate formation.

Reduction Prevents Conjugate Formation

Conjugation chemistry for the cross-linking or conjugating of proteins is a fairly well defined art. When formaldehyde is used to couple or cross-link proteins, the procedure may proceed by one of two routes: the Mannich reaction or via an immonium intermediate. The Mannich reaction consists of the condensation of formaldehyde (or some other aldehyde) with ammonia in its salt form, a primary or secondary amine, or even with amides, and another compound containing an active hydrogen. When conjugation occurs through an immonium intermediate, a primary amine may react with formaldehyde to form a quaternary ammonium salt. This intermediate spontaneously reacts to create a highly active immonium cation with loss of one molecule of water. The immonium cation is reactive toward nucleophiles in proteins and other molecules, including amines, sulfhydryls, phenolic groups, and imidazole nitrogens. The reaction yields methlene bridges between two nucleophiles, binding macromolecules with a one-carbon linker. Formaldehyde-facilitated conjugation reactions between molecules that both contain nucleophiles probably occur primarily by the immonium ion pathway, since the Mannich reaction proceeds at a slower rate. Nevertheless, both reaction pathways may simultaneously occur, especially when the reaction takes place at room temperature or greater.

An immonium intermediate is a common feature of both proposed cross-linking mechanisms and is one intermediate that would be susceptible to destruction by sodium borohydride. The Example below evaluated whether the time and temperature conjugation reaction of the present invention was occurring through a definable chemical pathway. Diphtheria toxoid, or a mixture of Dt and rCtB, was reduced with sodium borohydride before these proteins were used in the time and temperature conjugation reaction to see if this treatment could prevent conjugation. As shown below, this treatment markedly reduces the amount of conjugate formed. These results indicated that an active constituent in the conjugation reaction is present in Dt and can be the proposed immonium intermediate. It should also be noted that an immonium intermediate would be in equilibrium with the free amine and formaldehyde. Either the immonium or formaldehyde could be reduced by sodium borohydride to prevent conjugation.

EXAMPLE 8

Five (5) time and temperature conjugation reactions (Samples A-E) were prepared and incubated for 8 weeks at 4°C as indicated below. Following the incubation period, each sample was submitted to Gm1 ELISA with detection for Dt as described in Example 1. Sample A: Control Mixture of Dt and rCtB. Diphtheria toxoid and cholera toxin B were mixed together to give final concentrations of 4.5 mg/ml and 1.5 mg/ml, respectively. Sample B: In Situ Reduction of Dt and rCtB. Diphtheria toxoid and rCtB were mixed together at the final concentrations of 4.5 mg/ml and 4.5 mg/ml, respectively. Solid sodium borohydride was added to a final concentration of 0.1 M and the reaction proceeded at room temperature. After 3 hours, the pH was checked and found to be approximately 8.0. The reaction was capped and stored at 4°C. Sample C: Control Mixture of Dt and rCtB. Diphtheria toxoid and rCtB were mixed together and diluted with 0.1 M sodium phosphate, pH 7.5, to give final concentrations of 1.7 mg/ml and 0.6 mg/ml, respectively. Sample D: Reduction and Purification of Dt. Diphtheria toxoid (5.4 mg/ml) was mixed with solid sodium borohydride (0.1 M). The reduction proceeded for 3 hours at room temperature. Salts and excess sodium borohydride were removed from the reaction by gel filtration on a G25 column run in 0.1 M sodium phosphate, pH 7.5. The protein containing fractions were pooled and mixed with rCtB at concentrations of 1.7 mg/ml Dt and 0.6 mg/ml rCtB. Sample E: On the day of the Gm1 ELISA, a fresh mixture of Dt and rCtB was prepared as a control for the ELISA. Diphtheria toxoid and rCtB were mixed together to give final concentrations of 4.5 mg/ml Dt and 1.5 mg/ml rCtB. All samples were immediately used in the Gm1 ELISA determination for Dt.

The results from this experiment are shown in Table I below. Comparing the control mixture results (Samples A and C) to the reduction samples (B and D) shows that sodium borohydride reduction prior to the time and temperature conjugation procedure reduces the yield of conjugates formed, as measured by relative ELISA Units (EU), by greater than approximately 5 fold. This reduction in conjugation formation suggests that an immonium intermediate is present in the conjugation reaction of the present invention.

Table
Sodium Borohydride Reduction Prior to Conjugation

	A	B	C	D	E
[Dt] mg/ml	4.5	4.5	1.7	1.7	4.5
[CtB] mg/ml	1.5	1.5	0.6	0.6	1.5
Reduction	none	in situ	None	Dt/purify	none
CtB-Dt Conjugates Bound to Gm1 (relative EU)	887	159	336	53	none detected

EXAMPLE 9
Stability of Conjugates

Stability problems exist for a variety of bacterial vaccines. To test the stability of vaccines produced using the time and temperature methods of the present invention, conjugates of Dt and rCtB were prepared by mixture of these two proteins in a 1:1 mass ratio. The final concentrations were 3.7 mg/ml Dt and 3.7 mg/ml rCtB. The mixtures were stored at 4°C for 2 months to allow conjugation to take place, and then aliquots of the conjugates were treated as described below.

One aliquot without further treatment was stored at 4°C as a control. Another aliquot was diluted 25-fold in PBS and stored at 4°C. Solid sodium borohydride was added to two aliquots of conjugate to a final concentration of 180 mM and the solutions were allowed to react for 3 hours at room temperature. The pH of the reactions was monitored with pH paper. One portion of sodium borohydride reduced material was transferred to 4°C; another portion was diluted 25-fold with PBS and then transferred to 4°C. Another aliquot was mixed with neat ethanolamine to achieve a concentration of 0.4 M. This sample was then incubated at 4°C. All samples were incubated as indicated for 1 month prior to analysis of the conjugates by Gm1 ELISA with detection for Dt. Gm1 ELISA detecting for Dt was performed as previously described in Example 1. The results from these assays are shown in FIGURE 12.

The preformed conjugates of Dt and rCtB, as detected by Gm1 ELISA, are essentially unaltered by dilution, reduction, or ethanolamine treatment. This result indicates that the earlier observation that sodium borohydride treatment of diptheria toxoid or mixtures of Dt and rCtB prior to conjugate formation lowers the yield of conjugation rather than destroying conjugates.

EXAMPLE 10
Purification of Dt-CtB Conjugates

Time and temperature dependent conjugates of Dt and rCtB were made and purified using the methodology described below. Diphtheria toxoid (SBL Vaccin AB; Sweden; Batch U237) was mixed with rCtB (SBL Vaccin AB; Sweden; Batch 65/96) at a mass ratio of 3:1, respectively. A total of 30 ml was prepared at the final concentrations of 4.52 mg/ml Dt and 1.5 mg/ml rCtB. This solution was stored at 4°C for 3 months.

The conjugated material was purified on a ganglioside affinity column. The column resin consisted of a solid phase resin to which a mixture of gangliosides had been covalently linked. A mixture of bovine brain gangliosides (100 mg) (Calbiochem, San Diego, CA; product 345718) was hydrolyzed in 2.25 ml methanol and 0.25 ml 10 M potassium hydroxide according to the methods of Tayot *et al.* (*Eur. J. Biochem.* 113: 249-258 (1981); hereby incorporated by reference) and Neuenhofer (*Biochem.* 24: 525-532 (1985); hereby incorporated by reference) to generate the corresponding lyso-gangliosides. The reaction was heated to 110°C for 5 hours and then neutralized by the addition of 150 µl neat acetic acid and dried under vacuum. The residue was dissolved in 2 ml water, dialyzed against deionized water for 3 hours in a 12,000-14,000 molecular weight cut off (MWCO) membrane and the remaining solution was dried under vacuum and used without further purification. The solid mixture of lyso-gangliosides was suspended in methanol and mixed with Affigel-10 resin (10 ml)(Bio-Rad; Hercules, CA). The solution was mixed by gentle rocking for 2 days at room temperature. Following this procedure, the supernatant was removed and the resin was washed with methanol. Any remaining activated sites on the resin were reacted with 100 mM ethanolamine in methanol for 30 minutes at room temperature. The resin was successively washed with methanol, water, and phosphate buffered saline. Free amines on the gangliosides were modified with acetic anhydride (to 0.15 M final concentration) added in small portions while maintaining the pH at 7 by the addition of small portions of 10 M KOH. The resin was washed in phosphate buffered saline (PBS) and transferred to an Econo-column (Bio-Rad; Hercules, CA) for use.

Conjugates of Dt and rCtB were purified from unconjugated material by affinity and size exclusion chromatography. A mixture of Dt and rCtB that had been mixed and incubated at 4°C for 3 months was centrifuged at 6500 rpm in an SS34 rotor for 15 minutes to remove any precipitates from the solution. The supernatant was drawn off and applied to a ganglioside column that had been equilibrated in PBS. The flow-through was saved and the column was then washed with 100 ml of PBS. Protein was eluted from the affinity column with 4 M guanidine thiocyanate and collected in fractions of 5 ml. Fractions 2, 3, and 4 were pooled and dialyzed in a 5000 MWCO cellulose acetate dialysis membrane against three 1 L changes of 0.1 M sodium phosphate, pH 7.5.

The initial conjugate preparation, the flow-through from the ganglioside affinity column, and the dialyzed and eluted products were analyzed by Gm1 ELISA for Dt-CtB conjugates described in Example 1. No conjugates capable of binding to Gm1 were found in the flow through from the affinity column.

To remove unconjugated rCtB from the affinity purified material, the sample was fractionated on a size exclusion column, Superdex 200pg (Pharmacia Biotech; Piscataway, NJ), run in PBS. The column eluate was monitored for absorbance at 280nm and each fraction was submitted to Gm1 ELISA with detection for Dt. Those fractions that had a shorter retention time than rCtB and that contained Dt as measured by Gm1 ELISA were pooled. The final protein solution was concentrated in an Amicon Ultrafiltration (Millipore Corp.; Bedford, CA) devise with a YM10 membrane to a concentration of 2 mg/ml as determined by Bio-Rad (Hercules, CA) protein assay.

This purification scheme was performed 4 times to generate enough material for animal immunization studies and analysis of the products. Aliquots from each step of the first purification were collected for analysis. The analysis of each sample indicated that a 6 fold purification of conjugates was achieved (See Table II).

Table II
Purification of CtB-Dt Conjugates

Step	Protein Yield (mg vs. IgG STD)	Dt-CtB Conjugates (Relative ELISA Units (EU))	Dt-CtB Conjugates / mg Protein (EU/mg protein)	Fold Purification
Initial	37.8	98,200	2,600	1
Supernatant	23.9	79,700	3,340	1.3
Affinity Eluate	26.0	228,900	8,800	3.4
FPLC Eluate	7.8	113,200	14,500	5.6
Final Concentration	7.5	121,200	16,200	6.2

Calculating from the purification data, up to 20% of the protein in the Dt-CtB mixture was isolated using this purification method. Since there was no loss of Dt-CtB as measured in the Gm1 ELISA, it was estimated that up to 20% of the initial protein was Dt-CtB conjugates.

EXAMPLE 11
Evaluation of the Immunogenicity of Purified rCtB-Dt Conjugates

To demonstrate the superior immunogenicity of rCtB-conjugate vaccines over the immunogenicity of the uncoupled component parts, conjugates of Dt and rCtB were made and purified from the unconjugated components as described in Example 10. Briefly, the rCtB-Dt conjugate mixture was passed over a Gm-1 affinity column. Unbound material was washed away and the bound material (rCtB-Dt conjugate) was eluted with 4 M guanidinium isothiocyanate at a neutral pH. Dialysis against PBS produced a product enriched ~6 fold in bioactivity as measured by detection of diphtheria antigen in a Gm-1 binding ELISA.

Dt and rCtB were mixed immediately prior to immunization at a 3:1 molecular ratio to achieve a final concentration of 2.1 mg/ml Dt and 0.7 mg/ml CtB. The desired dose was 25 μg Dt and 8.3 μg rCtB per mouse. SPDP conjugated vaccine (Group 7) consisted of material isolated by FPLC and identified as the Gm-1 binding high molecular mass fractions as described in Example 1. The SPDP preparation was not affinity purified. Test animals were immunized three times biweekly by either intranasal (i.n.) or intramuscular (i.m.) route. Ten animals in each group were immunized as described in Table III.

Table III

Immunization Schedule

Group No.	Times of Immunization	Route	Vaccine Composition
1	3 x biweekly	i.n.	0.125 μg rCtB-Dt in 25 μg Dt + 8.3 μg rCtB
2	3 x biweekly	i.n.	0.6 μg rCtB-Dt in 24.4 μg Dt + 8.1 μg rCtB

3	3 x biweekly	i.n.	3.0 μg rCtB-Dt in 22 μg Dt + 7.3 μg rCtB
4	3 x biweekly	i.n.	15.0 μg rCtB-Dt in 10 μg Dt + 3.3 μg rCtB
5	3 x biweekly	i.n.	3.0 μg rCtB-Dt in PBS
6	3 x biweekly	i.n.	Fresh mixture of 25 μg Dt + 8.3 μg rCtB
7	3 x biweekly	i.n.	12 μl SPDP conjugate (25 μg Dt: 8.3 μg rCtB)
8	3 x biweekly	i.m.	25 μl duplex

All animals were bled 10 days following each immunization and every 2 weeks thereafter. The samples were analyzed for their serum IgG titer using the ELISA technique described in Example 1. The results of this analysis are shown in FIGURE 13. The 15 μg of time and temperature conjugated rCtB-Dt vaccine compositions was significantly more immunogenic than preparations of SPDP conjugate or than mixtures of Dt with rCtB containing little or no conjugate.

These results show that the concentration of the time and temperature conjugates is directly related to an increase in systemic immunogenicity of the vaccine preparation. This conclusion is supported by the observation that animals immunized with increasing concentrations of time and temperature conjugates showed a dose dependent increase in the lung tissue IgA response. Accordingly, Dt is more immunogenic when coupled by the time and temperature dependent methodology of the present invention to rCtB than when merely mixed together or when coupled using SPDP.

EXAMPLE 12
rCtB-Dependent Promotion of Vaccine Composition Immunogenicity

Vaccine conjugates were prepared over 4 weeks at various rCtB-Dt molecular ratios to study the effect of molecular ratio on the observed immunogenicity of the target antigen. Vaccine compositions were prepared containing Dt alone (Dt without rCtB), and rCtB-Dt ratios of 1:6, 1:3 and 1:1 (equimolar). To measure the effect of rCtB on the immunogenicity of the Dt immunogen, each test group received the same concentration of Dt (25 μg), with or without rCtB, by intranasal administration. Accordingly, groups of 10 Balb/c mice were immunized 3 times biweekly with vaccine preparations. Two control groups received intramuscular DUPLEX or intranasal SPDP vaccine prepared at equimolar concentrations of Dt and rCtB.

The results for this testing are shown in FIGURE 14. The data represent the geometric mean serum IgG endpoint titer ± the standard error of the mean. The results show that serum IgG response to Dt was correlated with an increasing molar ratio of rCtB:Dt. Although all animals received the same amount of Dt immunogen (25 μg of Dt), those animals receiving the immunogen and rCtB responded more effectively. This result shows that rCtB is acting as a carrier and/or an adjuvant to promote anti-Dt serum IgG immune responses.

The IgA and IgG responses for the various vaccine compositions were also assayed by ELISA on the lung fluids of the experimental animals. The results from these assays are shown in FIGURE 15. As in the serum, the upper and lower respiratory tract contained elevated levels of anti-Dt IgG and IgA following vaccination with rCtB-Dt

conjugated using the method of the present invention. Thus, intranasal immunization with time and temperature formaldehyde conjugated rCtB-Dt stimulated increased levels of anti-Dt IgG in the serum and both IgG and IgA in the respiratory mucosal tissues. In contrast, the commercial vaccine composition (DUPLEX) stimulated only IgG in the serum and mucosa. The SPDP conjugated vaccine compositions stimulated a less significant immune response.

The results from these experiments show that the time and temperature rCtB-Dt conjugates of the present invention are as effective in the induction of serum and mucosal Dt specific IgG and more effective in the induction of Dt specific IgA in the lungs of recipient animals than are commercial intramuscular DUPLEX vaccine or intranasal vaccination with SPDP rCtB-Dt conjugate. Further, the molecular ratio of Dt to rCtB directly influenced the potency of the anti-diphtheria response.

Time and Temperature Conjugated rCtB Based Vaccines

The time and temperature dependent conjugation method of the present invention contemplates utility in generating rCtB based vaccine compositions directed against a number of pathogens in addition to diphtheria. Contemplated pathogens include: the herpes viruses; human immunodeficiency virus (HIV); hepatitis B virus; *Mycobacterium leprae*; *Escherichia coli*; *Staphylococcus* spp., such as *S. aureus*; *Streptococcus* spp., such as *S. pneumoniae*, *Helicobacter pylori*; *Chlamydia trachomatis* (Chlamydia); *Neisseria gonorrhea* (gonorrhea); *Treponema pallidum* (syphilis); *Hemophilus ducreyi* (chancroid); and *Trichomonas vaginalis* (trichomoniasis). Protection against sexually transmitted cancer associated viruses such as certain strains of human papilloma virus are also contemplated as target pathogens. Other pathogens include: influenza viruses; the rhinoviruses; *Mycobacterium tuberculosis* (tuberculosis); respiratory syncytial virus; rotavirus; *Corynebacterium diphtheriae* (diphtheria); *Bordetella pertussis* (pertussis); Japanese encephalitis; measles; rubella; mumps; and other pathogenic organisms that gain entrance to a host organism through its mucosal membranes.

EXAMPLE 13

Preparation and Evaluation of a Time and Temperature Conjugated rCtB- *Bordetella pertussis* Vaccine Composition

The time and temperature conjugation methodology of the present invention can also be applied to whole killed bacterial cells to enhance the mucosal immune response to killed cell vaccines. To illustrate this statement, a rCtB- *Bordetella pertussis* vaccine composition was generated using the time and temperature conjugation method of the present invention. To generate an efficient vaccine composition, 10^9 killed *B. pertussis* cells were conjugated to rCtB at the molecular ratio of 20,000, 100,000 and 500,000 molecules of rCtB per bacterium using the methods of the present invention.

Antigenic analysis of fixed *B. pertussis* after conjugation and extensive washing indicated that killed bacteria bound rCtB molecules in concentrations commensurate with the original conjugation ratios. This conclusion is supported by the data shown in FIGURE 16A, which compares the final concentration of rCtB after conjugation with the initial number of rCtB molecules per *B. pertussis* cell. Further, as seen in FIGURE 16B, a vaccine composition prepared using the time and temperature conjugation method of the present invention (solid bars) was more efficient at

linking rCtB to the targeted bacterium when compared to merely mixing rCtB and *B. pertussis* (Admixture; open bars), or by SPDP (shaded bars) conjugation.

Whole, killed *Bordatella pertussis* concentrate containing 10^{11} *B. pertussis* cells/ml in phosphate buffered saline, pH 6.9, was purchased from Michigan Biologic Products Institute (Lansing, MI). The pertussis cell concentrate was incubated with 0.1% formaldehyde at 22°C for 20 hours. Excess formaldehyde was then removed from the solution by dialysis into phosphate buffered saline, pH 7.2. Portions of the formaldehyde modified pertussis cells were mixed with rCtB at the following three concentrations: A: 7.2×10^{10} cells/ml with 3.3 mg/ml rCtB; B: 9.3×10^{10} cells/ml with 0.85 mg/ml rCtB; and C: 9.8×10^{10} cells/ml with 0.18 mg/ml rCtB. These concentrations are approximately equal to 500,000 molecules of rCtB per cell, 100,000 molecules of rCtB per cell, and 20,000 molecules of rCtB per cell, respectively. The samples were then incubated at 4°C for several weeks.

Conjugates of pertussis cells and rCtB were assessed after purifying free CtB from the conjugates by a series of pelleting and washing steps. Portions of the conjugated rCtB-pertussis mixtures and for comparison portions of freshly mixed solutions of rCtB and pertussis were subjected to the following protocol. A sample of each solution (250 µl) was centrifuged at 14,000 rpm in an EPPENDORF microcentrifuge (Brinkmann Instruments, Inc.; Westbury, NY) for 1 minute to pellet the bacteria and any associated rCtB. The supernatant was removed, and the pellet was suspended in 500 µl PBS. The solution was pelleted and washed with 500 µl PBS twice more. The final pellet was suspended into 250 µl PBS and prepared for analysis by ELISA as described generally in Example 1.

For ELISA, a portion of the cell suspension was prepared as a dilution series in 0.05 M sodium carbonate, pH 9.6, and adsorbed to a 96 well ELISA plate. For quantification, a portion of rCtB was prepared as a standard and adsorbed to the 96 well plate along side of the samples. Each well received 100 µl sample. The plate was sealed with a piece of plastic adhesive and incubated at 37°C overnight. The plate was then washed twice with PBS. To block, 100 µl of 1% BSA and 5% fetal bovine serum was added to each well and incubated at 37°C for 45 minutes. The plate was again washed twice with PBS. To detect the amount of CtB that co-purified with the *Bordatella pertussis* cells, a 1/2000 dilution of sheep anti-CtB (SBL Vaccin AB; Sweden) in 0.1% BSA in PBS was incubated for 2 hours at 30°C. The plate was washed five times with PBS. Then, horse radish peroxidase modified donkey anti-sheep Ig was added at a dilution of 1/2000 in 0.1% BSA in PBS. After incubation for 1.5 hours, the plate was washed five times with PBS. Finally, the plate was developed with the horse radish peroxidase substrate ortho-phenylene diamine as described in Experiment 1. The amount of rCtB bound to the plate was determined by comparison to the standard curve of rCtB in this assay.

The ratios of CtB to *B. pertussis* cells selected for preparation translate into the following amounts of each component per mouse dose:

Stoichiometry	CtB dose	Pertussis dose
500,000 CtB/cell	40 µg	0.9×10^9 cells
100,000 CtB/cell	8 µg	0.9×10^9 cells

20,000 CtB/cell	1.6 μg	0.9 x 10^9 cells

The dose of *B. pertussis* used in the immunizations per mouse is described in the literature as being an immunogenic dose. Also, the dose range of CtB 1.6 to 40 μg, is within the range known to stimulate an immune response in mice.

To evaluate the immunogenicity of these compositions, groups of C57Bl/6 mice were immunized intramuscularly with either whole cell DPT or DtaP from Pasteur Merieux Connaught (Swiftwater, PA) or immunized intranasally with rCtB conjugates prepared by SPDP conjugation at 100,000 rCtB/bacterium or with the time and temperature dependent conjugates of the present invention prepared at 20,000, 100,000, or 500,000 molecules rCtB/bacterium. The lung tissue immunoglobulin response to these immunogens is shown in FIGURE 17.

Animals receiving intranasal whole cell pertussis (WCP) alone induced low levels of both IgA (open bars) and IgG (shaded bars) in the lungs of vaccinated animals. Intranasal immunization with 20,000 rCtB/cell composition induced higher levels of IgA when compared to the response induced by WCP alone, however the IgG responses from these compositions were similar. In contrast, the 100,000 rCtB/cell vaccine composition induced a greater level of both IgA and IgG than any other composition tested. The amount of IgG and IgA induced by mixed but unconjugated rCtB at 100,000 molecules/bacterium was higher than that of either the WCP or 20,000 molecules/bacterium compositions, but less than that of the conjugated composition. The varied immunogenicity was especially noticeable in the IgA response observed. Low IgA responses were seen in animals immunized with the SPDP conjugated composition. No pertussis specific IgA was detected in the lungs of DTP or DTaP vaccinated animals. The IgG responses induced by the SPDP, DtaP, and DTP compositions varied, although each was higher than the WCP alone compositions.

Compositions containing 500,000 molecules/bacterium induced a much lower response to pertussis than did those containing 100,000 molecules/bacterium (CVP-100).

EXAMPLE 14

Time and Temperature Conjugated rCtB-Influenza Vaccine Composition

A conjugated rCtB-influenza vaccine was produced by taking purified influenza A/X-31 (H3N2) virus and inactivating it with formalin. Virus was used at a concentration of 1 mg/ml and treated with 0.025% formaldehyde for 3 days at 4°C. The virus was then dialyzed into 0.15 M sodium chloride, 0.25 mM calcium chloride, 0.83 mM magnesium chloride, 20 mM sodium borate, pH 7.2. To prepare conjugates with rCtB, rCtB was also dialyzed into this same buffer. Influenza virus was mixed with CtB at the following concentrations: 1 mg/ml influenza virus with 75 μg/ml rCtB; and 1 mg/ml influenza virus with 7.5 mg/ml CtB. The solutions were stored at 4°C for 2 months. The solutions contained approximately 1000 molecules of rCtB per virus particle or 100,000 molecules of CtB per virus particle, respectively.

The ratios of CtB to influenza virus selected for preparation translate into the following amounts of each component per mouse dose:

Stoichiometry	CtB dose	Influenza dose
1,000 CtB/cell	0.15 μg	2 μg
100,000 CtB/cell	15 μg	2 μg

Optimally immunogenic molecular ratios will vary for each vaccine target and can be determined using standard techniques known to those of skill in the art. The foregoing example illustrates such a technique.

EXAMPLE 15
Time and Temperature Conjugated rCtB-Herpes Simplex Virus I Vaccine Composition

The generation of a vaccine against the herpes simplex virus type I (HSV-1) is performed, where rCtB is conjugated to HSV-1 particles according to the method of Example 14.

EXAMPLE 16
Time and Temperature Conjugated rCtB-Herpes Simplex Virus II Vaccine Composition

The generation of a vaccine against the herpes simplex virus type II (HSV-2) is performed, where rCtB is conjugated to HSV-2 particles according to the method of Example 14.

EXAMPLE 17
Time and Temperature Conjugated rCtB-Rhinovirus Vaccine Composition

The generation of a vaccine against the rhinovirus is performed, where rCtB is conjugated to rhinovirus particles according to the method of Example 14.

EXAMPLE 18
Time and Temperature Vaccine Composition Induced Protection Against Lethal Challenge

Intranasal immunization with the time and temperature dependent rCtB-Dt conjugates of the present invention were used to protect guinea pigs from a lethal exposure to diphtheria toxin. Two groups of five Duncan-Hartley guinea pigs (~300 g) were exposed to 160 ng of subcutaneous diphtheria toxin ten days following three biweekly intranasal immunizations with PBS or 500 μg Dt alone. Three groups of ten animals were immunized 3 x biweekly with either intramuscular DUPLEX™ (Dt) vaccine at 1/6 human dose, or intranasal time and temperature dependent rCtB:Dt (1:3) composition at 166 μg or 500 μg. These animals were challenged ten days following their last immunization with 1.6 μg (10xMLD) toxin subcutaneously (s.c.).

FIGURE 18 shows the survival curves for animals in this study. All animals receiving 3 x 500 μg of the time and temperature conjugated rCtB:Dt vaccine composition survived the challenge by 10 MLD (1.6 μg) of s.c. diphtheria toxin. In contrast, only 40% of the animals receiving 3 x 500 μg of intranasal diphtheria toxoid alone survived 1 MLD (0.16 μg) of toxin, whereas no animals survived 1 MLD without vaccination. These results clearly show that the time and temperature conjugated vaccine compositions of the present invention protect immunized subjects from lethal challenge with diphtheria toxin.

Conclusion

We have discovered a method for conjugating carrier and immunogenic vaccine components in a time and temperature dependent manner. The conjugation of these components leads to vaccine compositions of enhanced immunogenicity.

Finally, the forgoing examples are not intended to limit the scope of the present invention, which is set forth in the following claims. In particular, various equivalents and substitutions will be recognized by those of ordinary skill in the art in view of the foregoing disclosure, and these are contemplated to be within the scope of the present invention.

WHAT IS CLAIMED:

1. A method of making a time and temperature cross-linked vaccine preparation comprising the steps of:

providing an immunogenic component and a carrier component; and

cross-linking said immunogenic component and said carrier component for no less than two weeks at a temperature of no more than 15°C.

2. The method of Claim 1, wherein said immunogenic component and said carrier component are cross-linked using an aldehyde selected from the group consisting of glutaraldehyde, formaldehyde, glyceraldehyde, acetaldehyde, phenylaldehyde, valeraldehyde, or 3,4-dihydroxyphenylacetaldehyde.

3. The method of Claim 1, wherein said immunogenic component and said carrier component are cross-linked using a ketone selected from the group consisting of acetone, methyl ethyl ketone, or 3-pentanone.

4. The method of Claim 1, wherein said immunogenic component is selected from the group consisting of *Bordetella pertussis* toxin subunit S2, S3, S4, S5, Diphtheria toxin fragment B, *E. coli* fimbria K88, K99, 987P, F41, CFA/I, CFA/II (CS1, CS2, CS3), CFA/IV (CS4, CS5, CS6), and P fimbraiae.

5. The method of Claim 1, wherein said carrier component is selected from the group consisting of cholera toxin, staphylococcal α-hemolysin toxin, the staphylococcal δ-hemolysin toxin, the *Vibrio cholerae* thermostable direct hemolysin toxin, the pertussis toxin, or the *E. coli* heat-labile enterotoxin.

6. The method of Claim 1, wherein said carrier component is a toxin subunit selected from the group consisting of the cholera toxin subunit A, the cholera toxin subunit B, and the *E. coli* heat-labile enterotoxin subunit B (LTB).

7. The method of Claim 1, further comprising a stabilizer.

8. The method of Claim 7, wherein said stabilizer is added prior to cross-linking said immunogenic component and said carrier component.

9. The method of Claim 7, wherein said stabilizer is added after cross-linking said immunogenic component and said carrier component.

10. The method of Claim 1, wherein said immunogenic component is diptheria toxin and the carrier component is cholera toxin subunit B.

11. The method of Claim 10, wherein said immunogenic component and carrier component are crosslinked using a formaldehyde solution for at least two weeks at a temperature of no more than 4°C.

12. The method of Claim **Error! Bookmark not defined.**2, wherein said immunogenic component is selected from the group consisting of viruses, bacteria, fungi, proteins, polypeptides, glycoproteins, lipids, glycolipids, or immunogenic portions thereof.

13. A vaccine preparation prepared by the method of any one of the foregoing claims.

14. The vaccine preparation of Claim 13 for use in generating an immune response in a mammal.

15. The vaccine of Claim 14, wherein the vaccine is for a mode of administration selected from the group consisting of nasal, oral, rectal, vaginal. inhalation and ophthalmic.

16. Use of the vaccine according to Claim 13 in the preparation of a medicament for generating an immune response in a mammal.

17. The use of Claim 16, wherein the medicament is for a mode of administration selected from the group consisting of nasal, oral, rectal, vaginal. inhalation and ophthalmic.

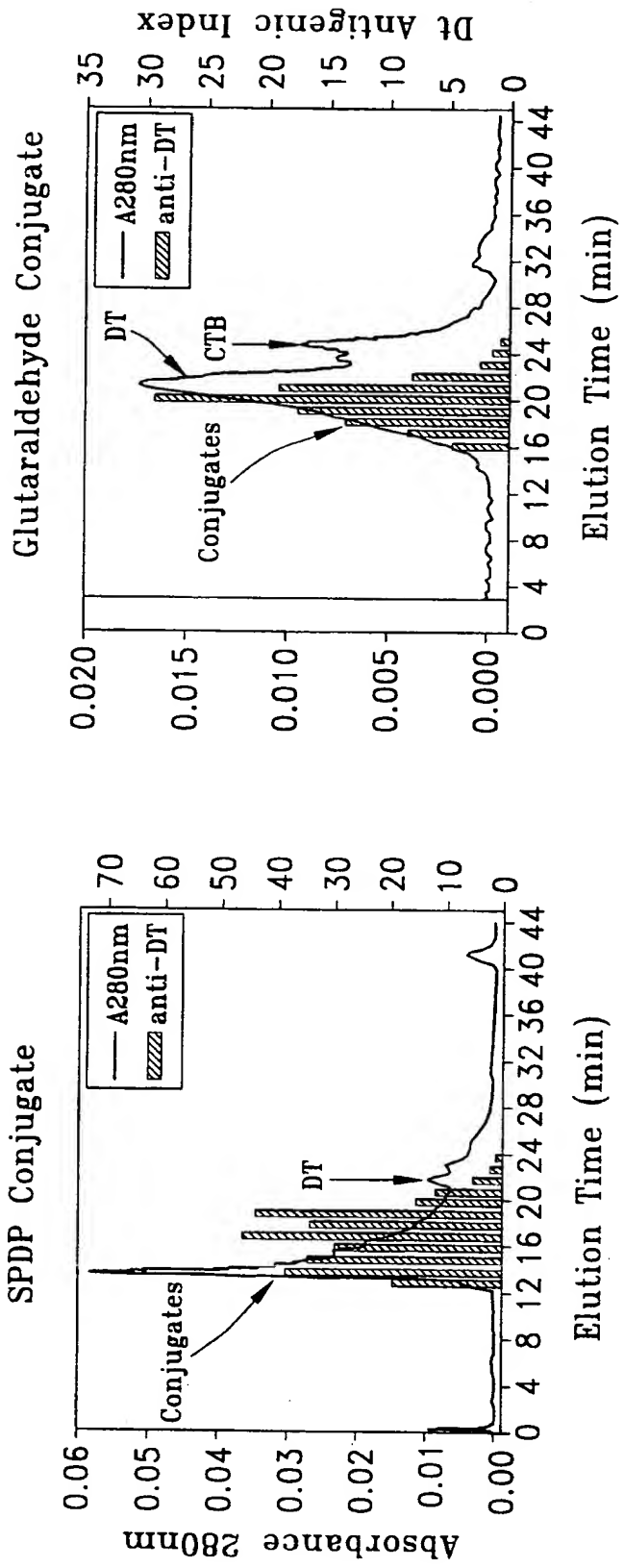

FIG. 1

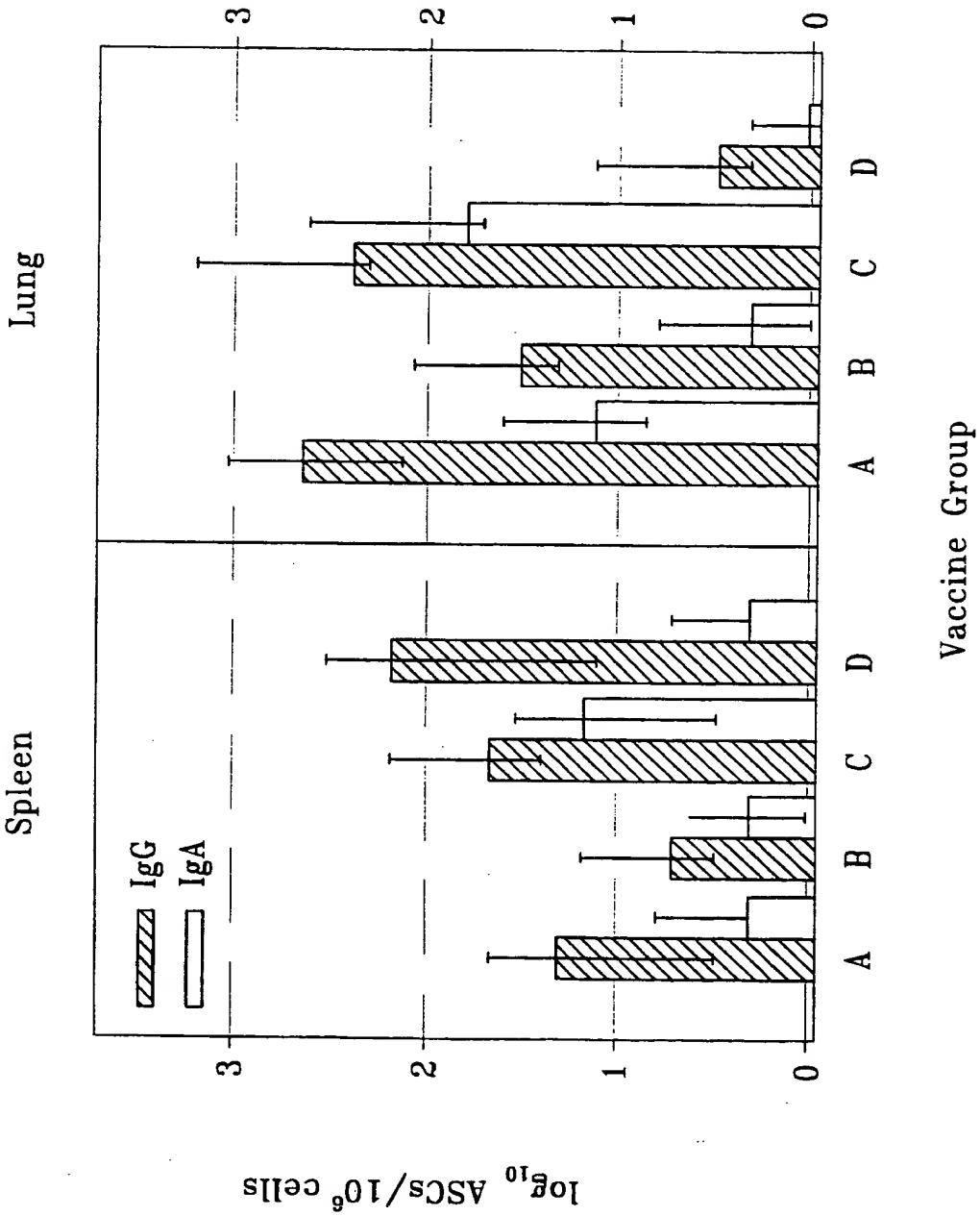

FIG. 2

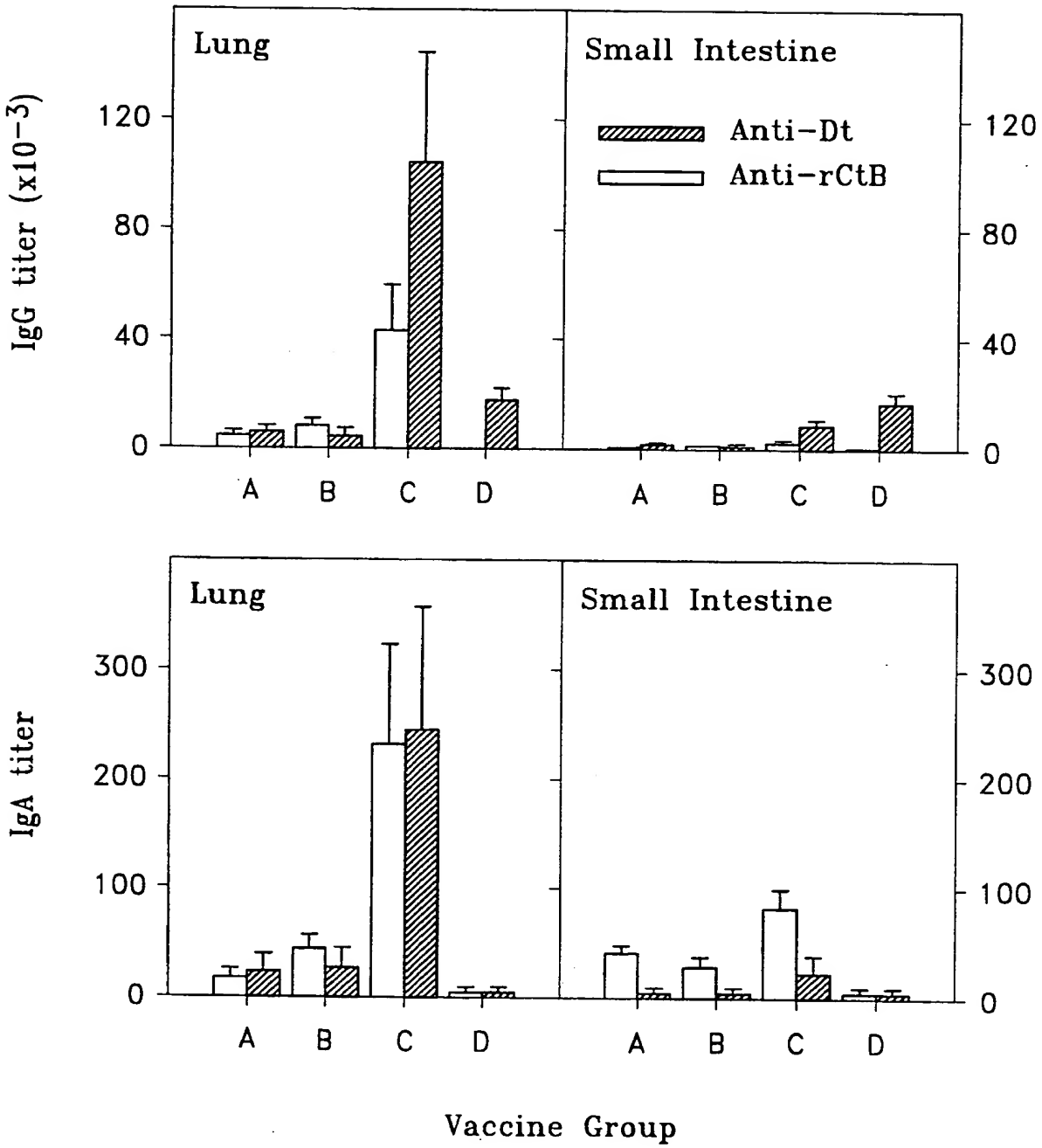

FIG. 3

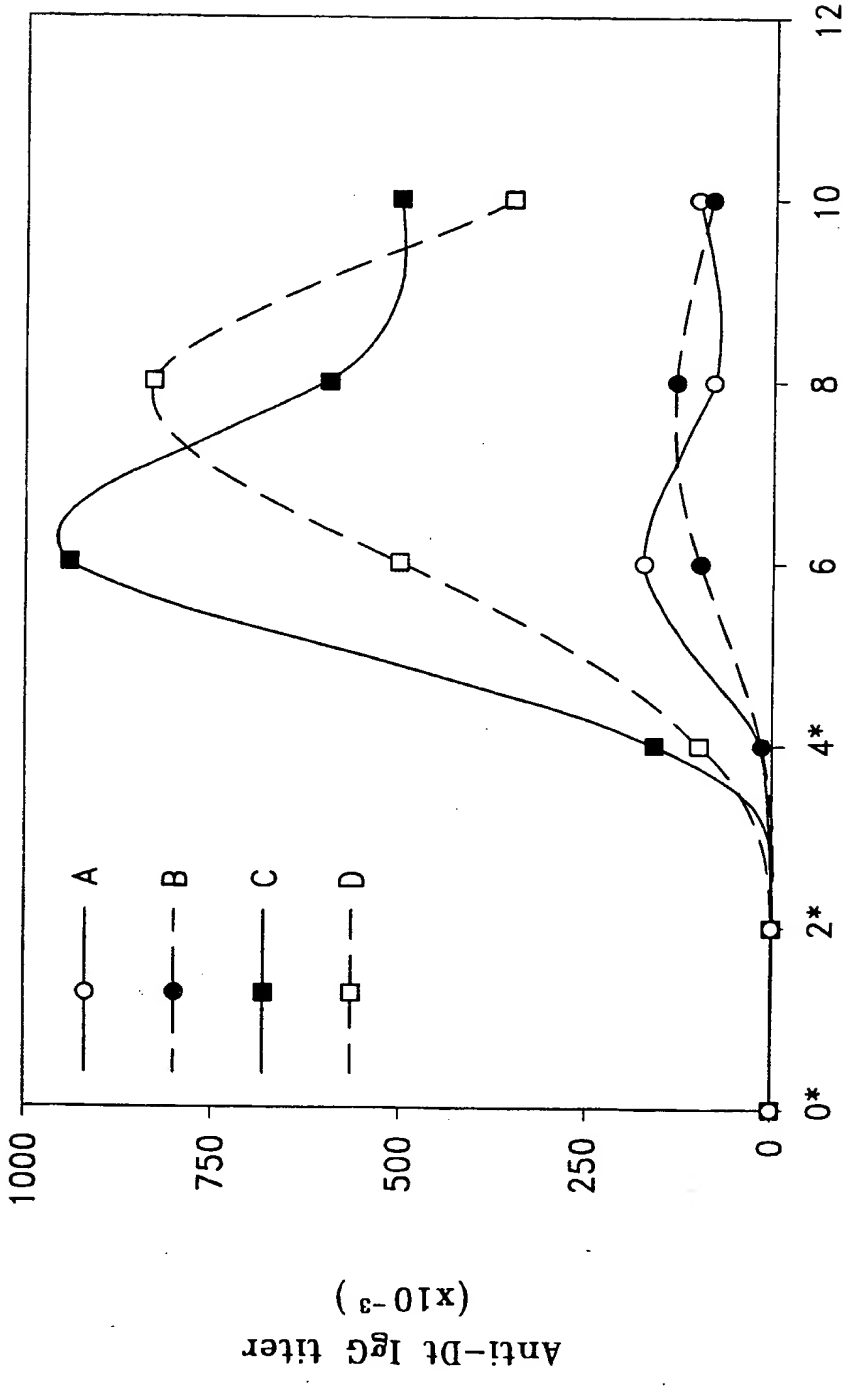

FIG. 4

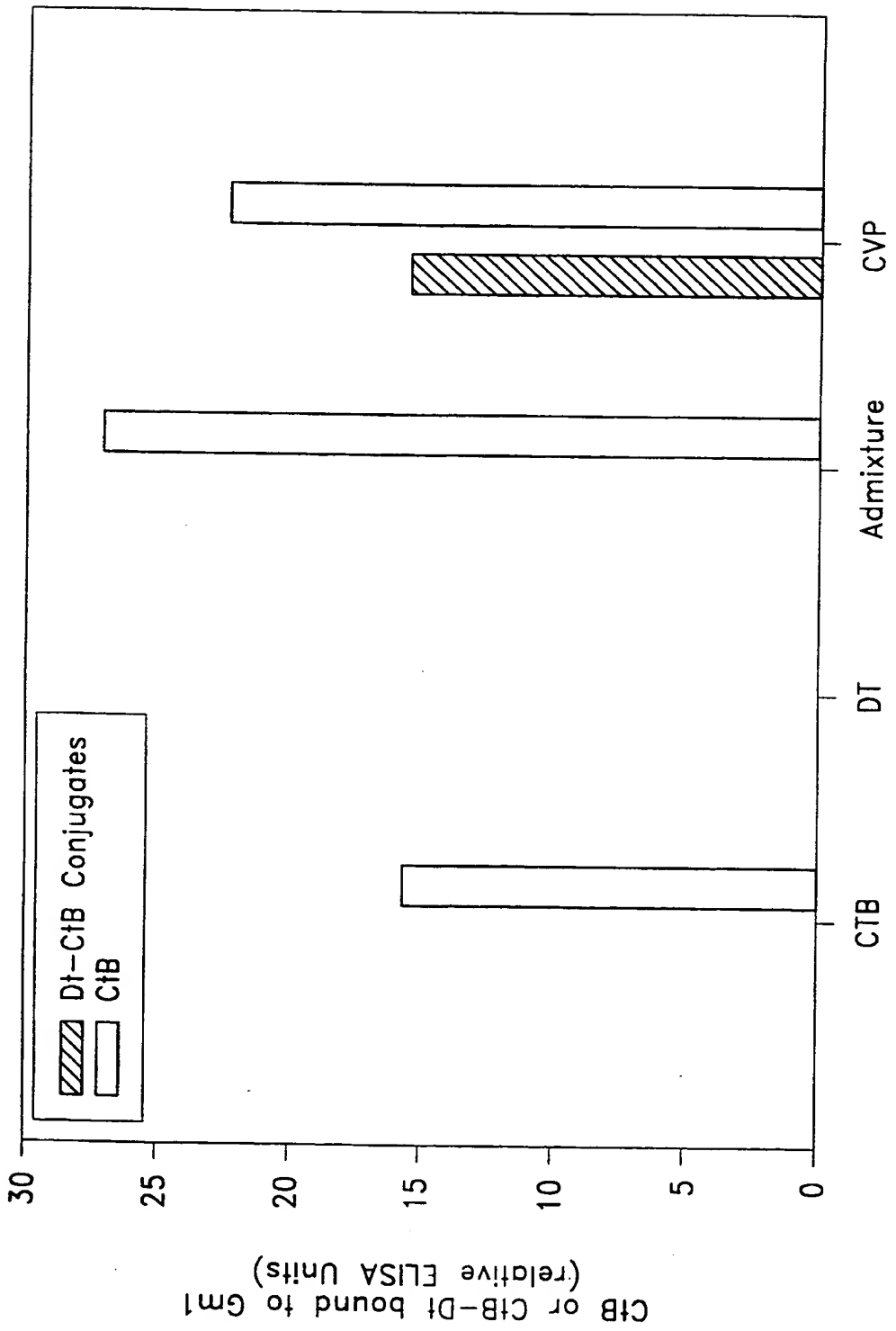

FIG. 5

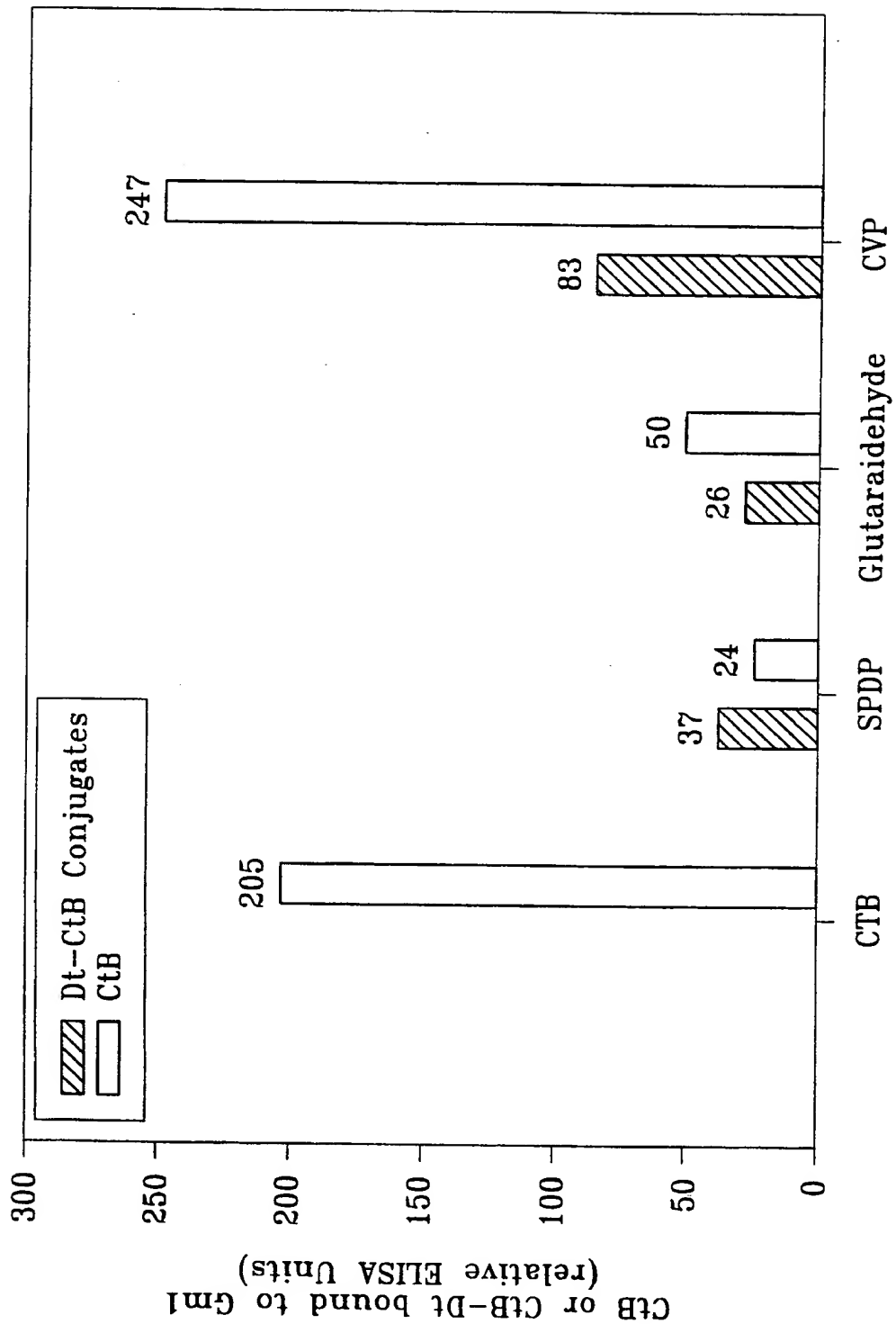

FIG. 6

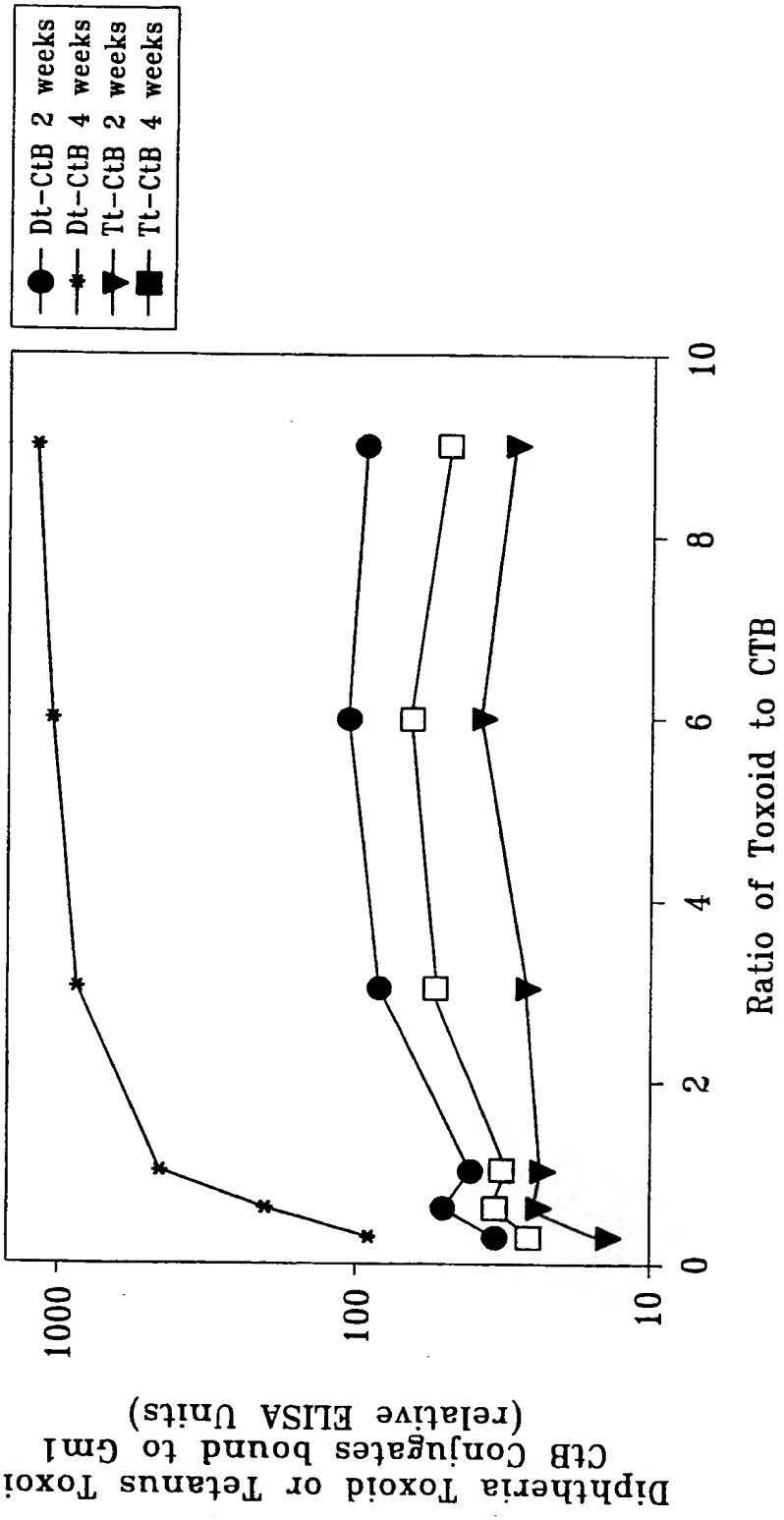

FIG. 7

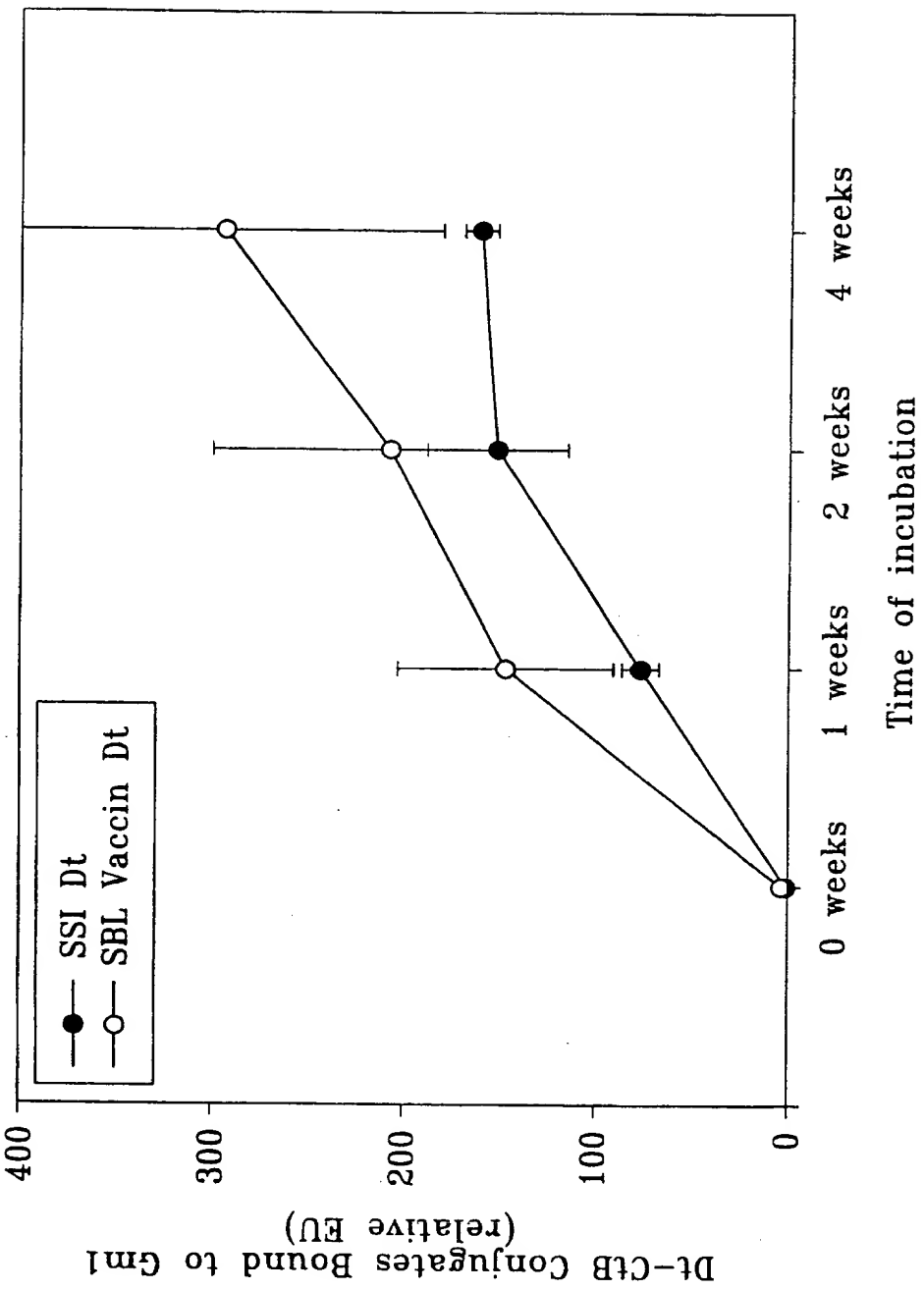

FIG. 8

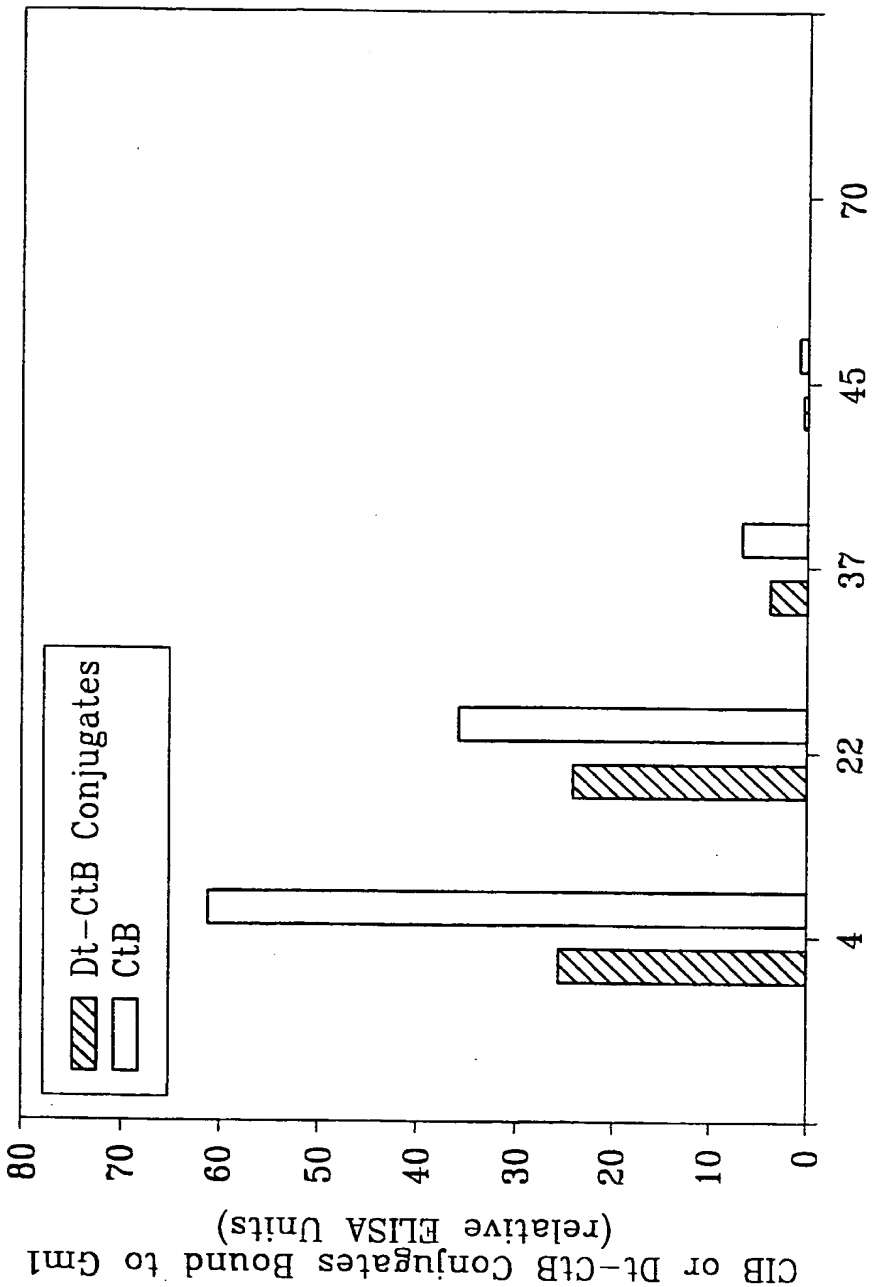

FIG. 9

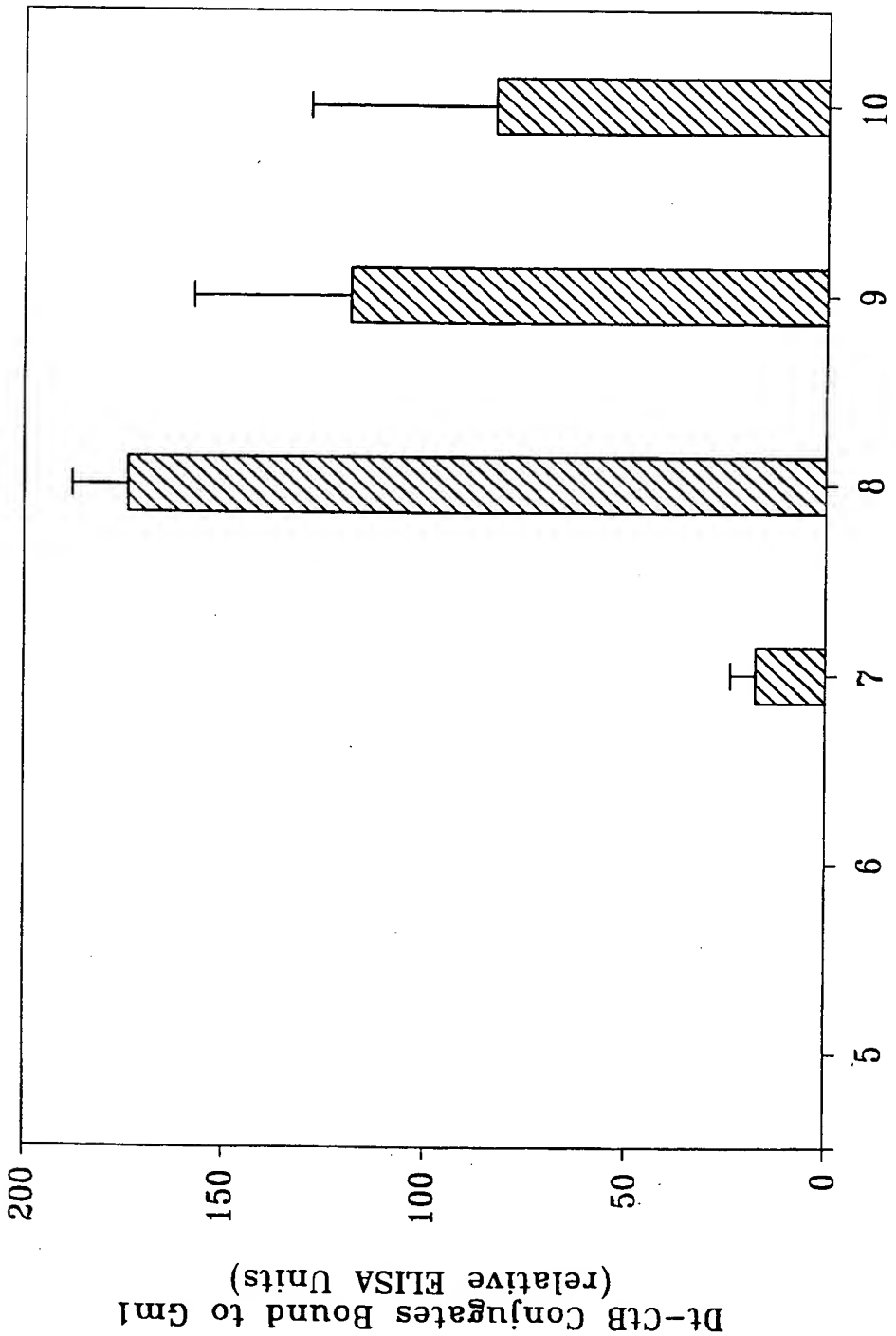

FIG. 10

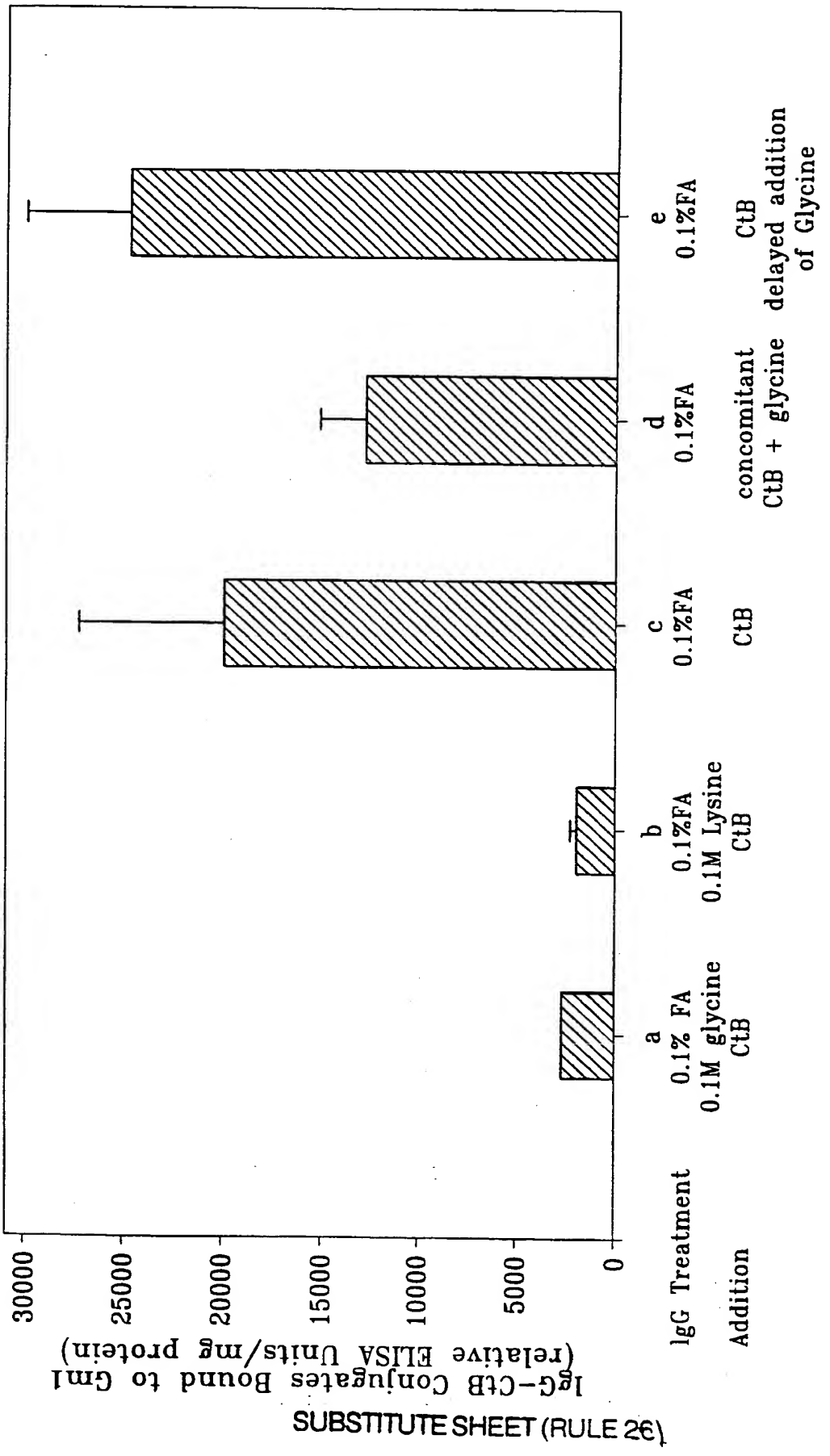

FIG. 11

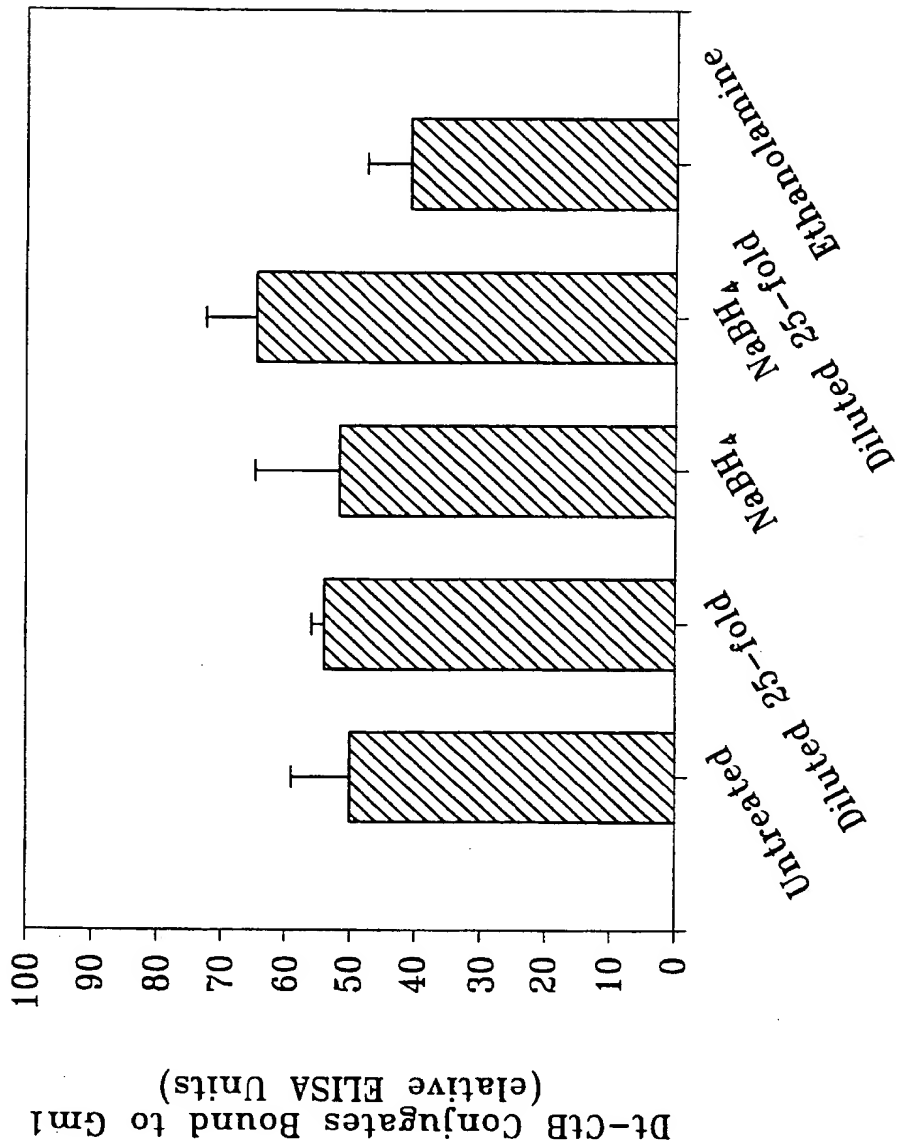

FIG. 12

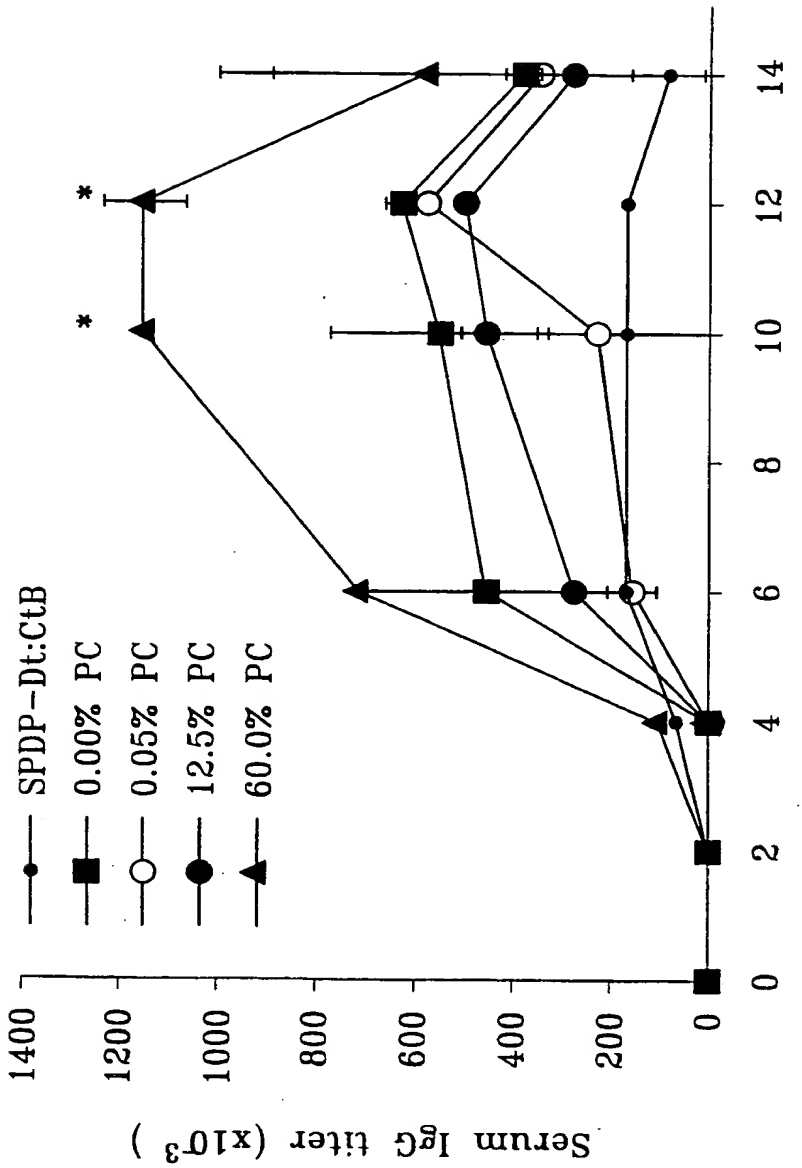

FIG. 13

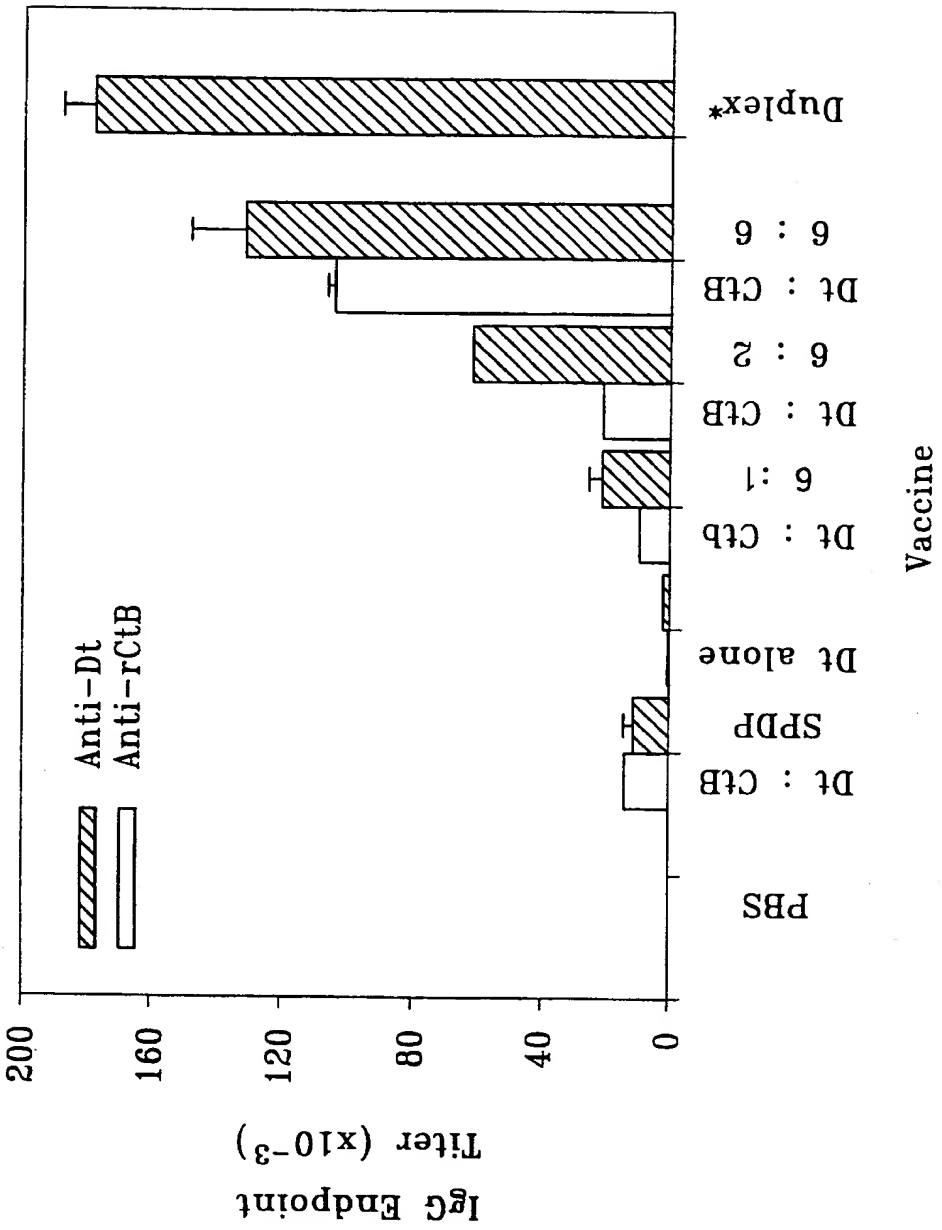

FIG. 14

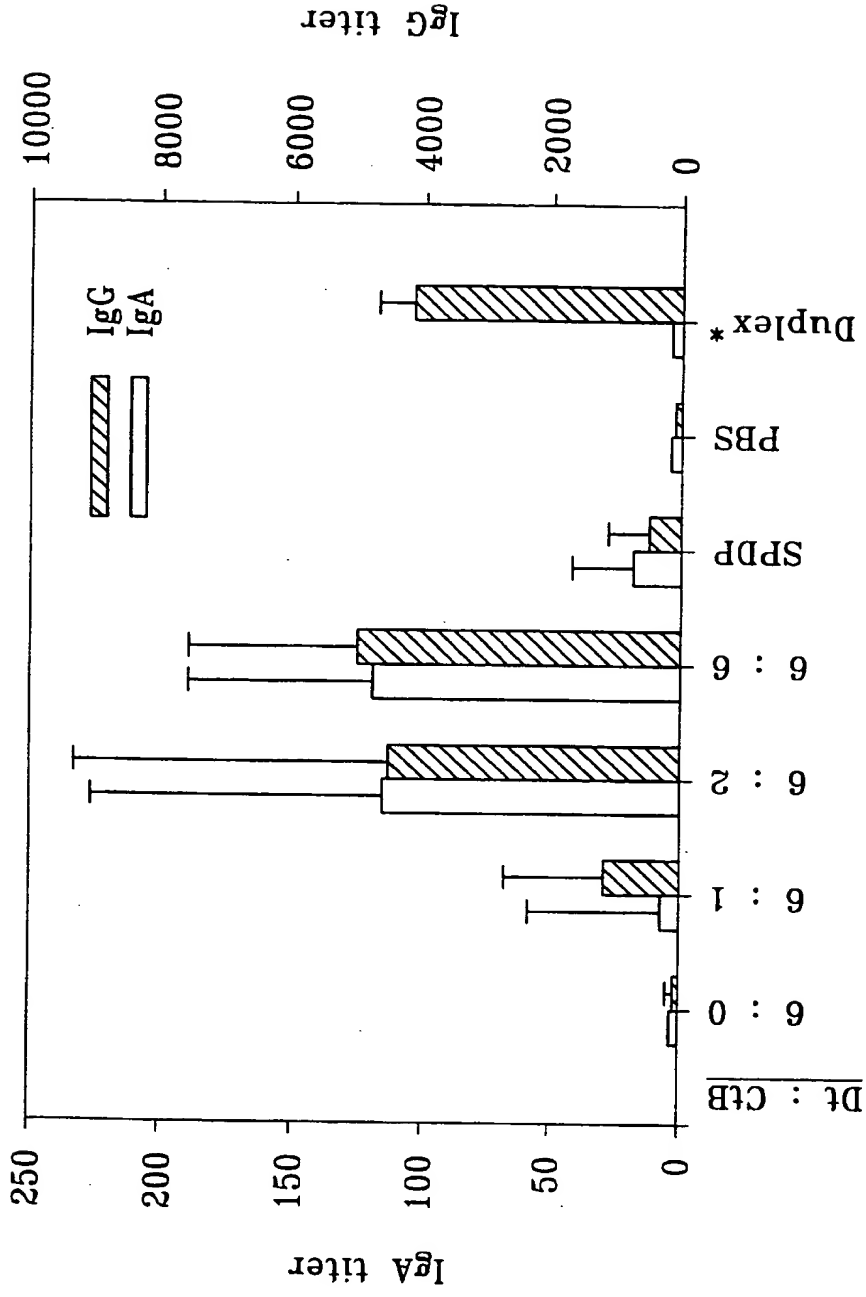

FIG. 15

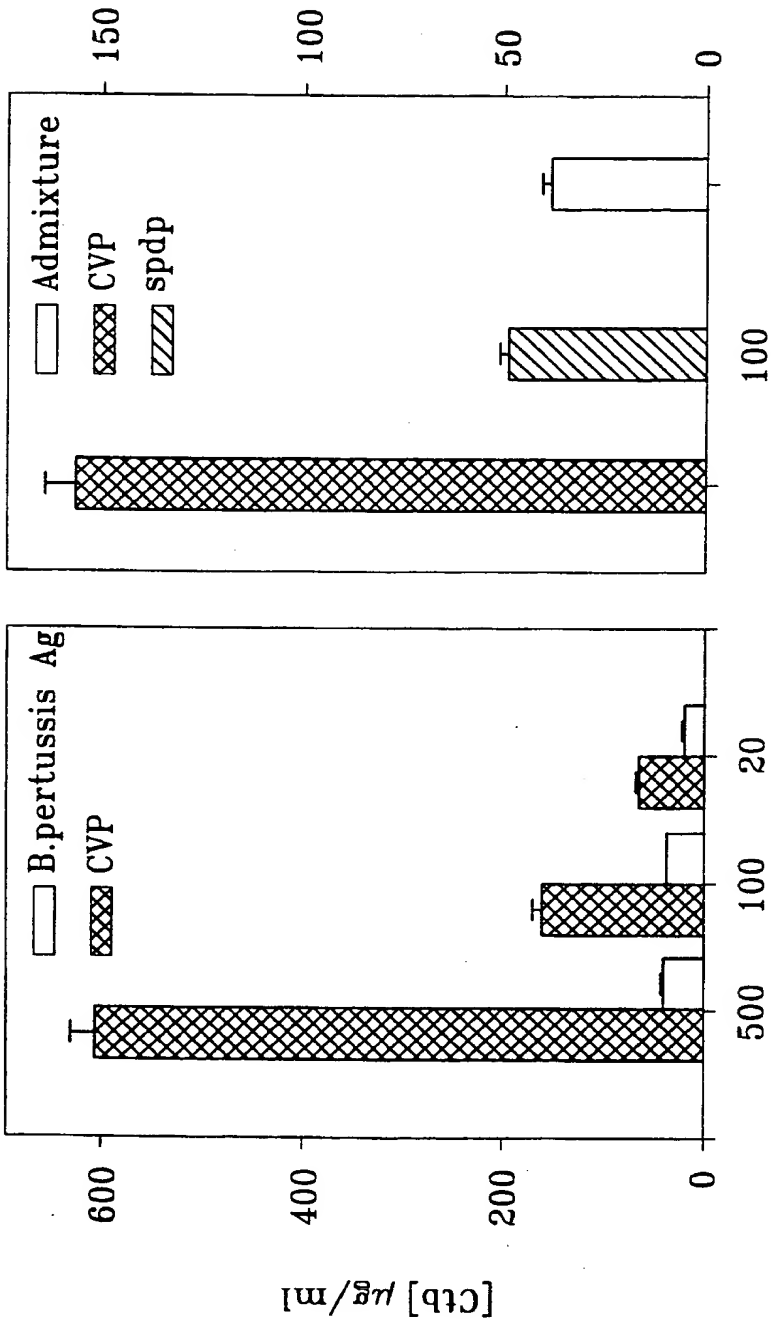

FIG. 16

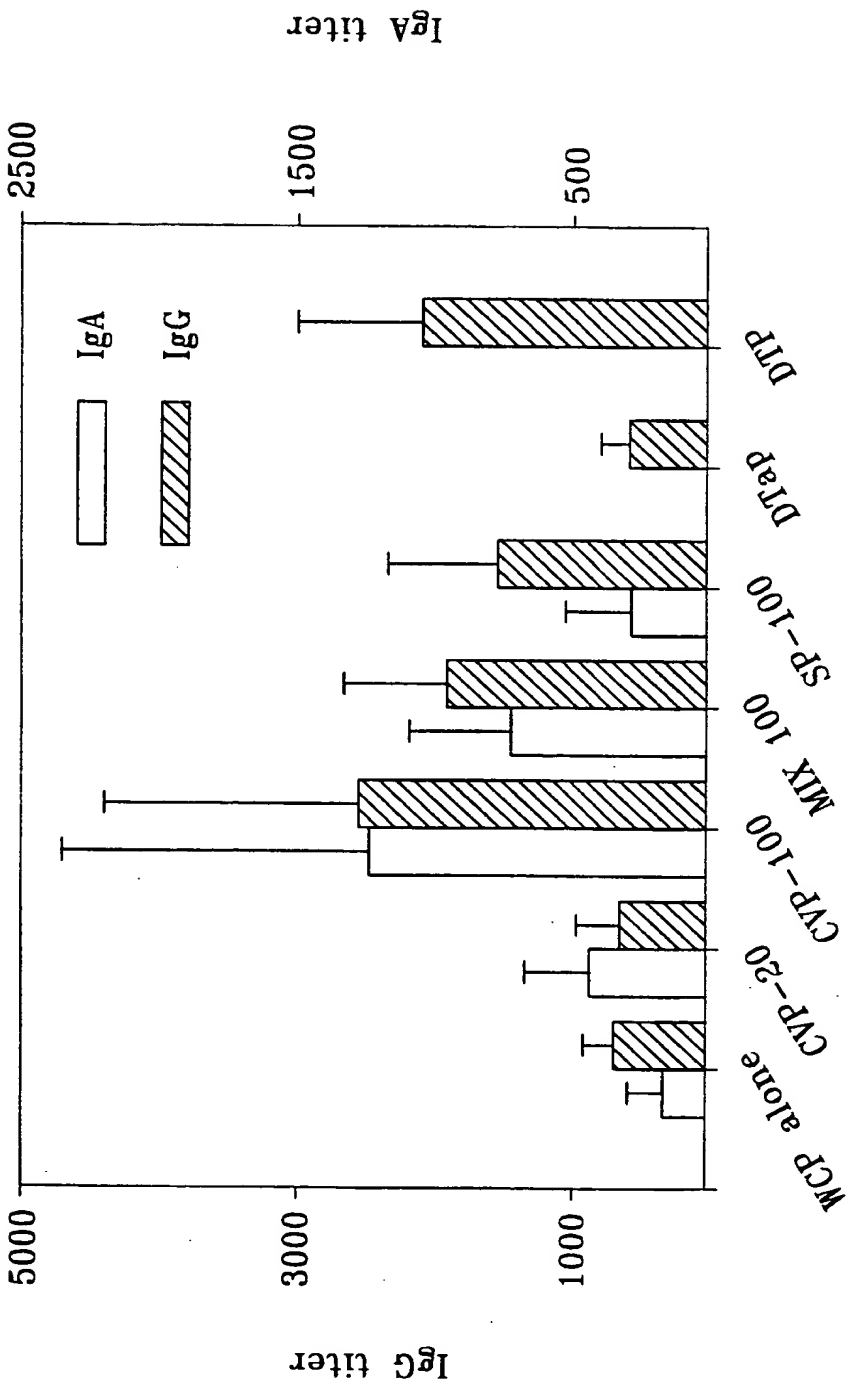

FIG. 17

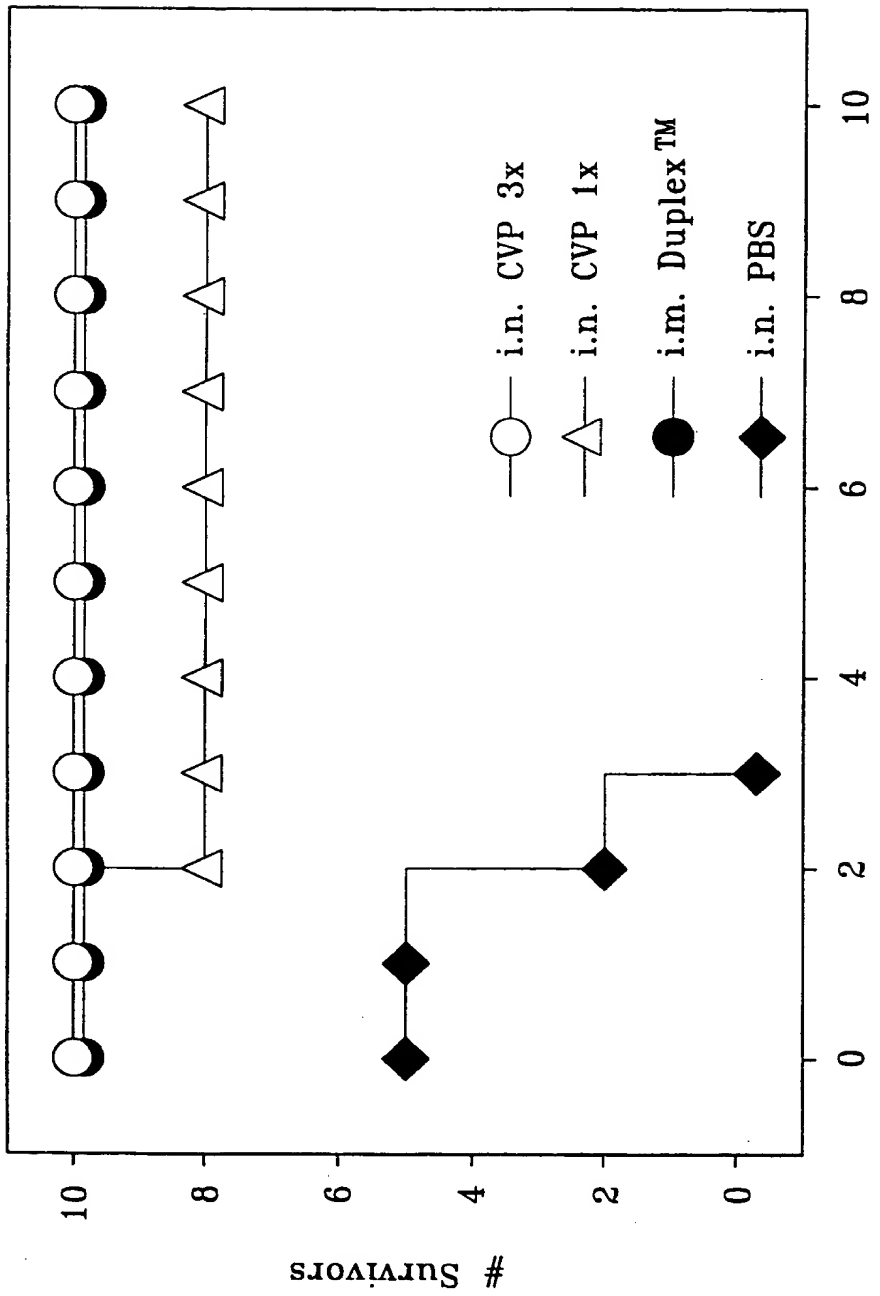

FIG. 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00943

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/193.1, 194.1, 197.11, 203.1, 234.1,236.1, 237.1, 238.1, 241.1, 242.1, 253.1, 261.1; 530/402

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,578,308 A (CAPIAU et al) 26 November 1996, especially column 5, lines 53-column 6, line 9.	1-17
A	US 5,453,273 A (WERNER et al) 26 September 1995, see entire document.	1-17
A	US 4,997,915 A (TAN et al) 05 March 1991, see entire document.	1-17

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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Date of the actual completion of the international search 10 MARCH 1999	Date of mailing of the international search report 31 MAR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JENNIFER GRASER Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/00943

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39/385, 39/116, 39/02, 39/05, 39/108, 39/10, 39/106; C07K 1/00

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/193.1, 194.1, 197.11, 203.1, 234.1,236.1, 237.1, 238.1, 241.1, 242.1, 253.1, 261.1; 530/402

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG, BIOSIS, MEDLINE, EMBASE, EUROPEAN PATENTS
search terms: cross link?, vaccin?, cold temperature, gluteraldehyde or formaldehyde or glyceraldehyde, ketone?, cholera? or staphylococc? or diphtheria, ctb